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**The role of ionotropic
glutamate receptors in memory
formation after
a passive avoidance task
in the domestic chick
(*Gallus domesticus*)**

**By
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A thesis submitted in partial satisfaction of the degree of Doctor of Philosophy.

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'Memory was the source of all identity, the only link in a flux of perceptions and events - not only for living beings but for the physical universe as well'

From "The Flies of Memory" a novel by Ian Watson ©1990, Victor Gollancz, London

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Publications based on research for this thesis

Papers

1. Stewart, M.G; Bourne, R.C and Steele, R.J. (1992) Quantitative autoradiographic demonstration of changes in binding to NMDA-sensitive [^3H]glutamate and [^3H]MK801, but not [^3H]AMPA receptors in chick forebrain 30 minutes after passive avoidance training. *Eur.J.Neurosci*, 4: 936 - 943.
2. Steele, R.J and Stewart, M.G. (1993) 7-Chlorokynurenate, an antagonist of the glycine binding site on the NMDA receptor, inhibits memory formation in day-old chicks (*Gallus domesticus*). *Behavioural and Neural Biol*, 60: 89-92.
3. Steele, R.J, Stewart, M.G and Rose, S.P.R. (1995) Increases in NMDA receptor binding are specifically related to memory formation for a passive avoidance task in the chick: a quantitative autoradiographic study. *Brain Res*, 674: 352-356.
4. Steele, R.J and Stewart, M.G. Involvement of AMPA receptors in maintenance of memory for a passive avoidance task in day old chicks (*Gallus domesticus*). *Eur J.Neurosci*, in press.
5. Steele, R.J, Dermon, C and Stewart, M.G. D-cycloserine causes transient enhancement of memory for a weak aversive stimulus in day old chicks (*Gallus domesticus*). *Behavioural and Neural Biol*, submitted.
6. Steele, R.J and Stewart, M.G. Quantitative Autoradiographic demonstration of alterations in binding of [^3H]glycine, NMDA-sensitive [^3H]glutamate and [^3H]MK801, to chick forebrain 3 h after passive avoidance training. *Brain Res*, submitted.
7. Steele, R.J and Stewart, M.G. Alterations in maximal [^3H]L-glutamate binding to N-methyl-D-aspartate receptors 3 and 6.5 hours after passive avoidance training of day old domestic chicks (*Gallus domesticus*). *Neurosci Lett*, submitted.

Abstracts

1. Stewart, M.G, Bourne, R.C.,and Steele, R.J. (1992) Passive avoidance learning causes an increase in binding to NMDA-sensitive [^3H]glutamate and [^3H]MK801, but not [^3H]AMPA receptors in chick forebrain. *Neurosci Letts*, S42, S9.
2. Steele, R.J, Bourne, R.C and Stewart, M.G. (1993) Temporal and spatial shift of changes in binding to excitatory amino acid receptors in chick forebrain following passive avoidance training. *Brain Res. Assoc. Abstr*; Vol 10, p 46.
3. Steele, R.J, Stewart, M.G and Bourne, R.C. (1993) The involvement of glycine and NMDA receptors in passive avoidance training in the domestic chick, *Gallus domesticus*. *Abst proc. No 104, 25th EBBS meeting, Madrid*.
4. Steele, R.J and Stewart, M.G. D-cycloserine causes transient enhancement of memory retention for a weak aversive stimulus when day old chicks (*Gallus domesticus*) are trained on a passive avoidance task. *Brain Res. Assoc. Abstr*; Vol 11, p 84, 1994.
5. Steele, R.J, Stewart, M.G. Demonstration of alterations in NMDA receptor kinetics following passive avoidance training in day old chicks (*Gallus domesticus*) using quantitative receptor autoradiography. *Brain Res. Assoc. Abstr*; Vol 11, p 84, 1994.
6. Steele, R.J, Stewart, M.G. Involvement of AMPA receptors in the maintenance of memory for a passive avoidance task in day old domestic chicks (*Gallus domesticus*). *Brain Res. Assoc. Abstr*; Vol 12, p 113, 1995.

Abbreviations

Anatomical

AA:	anterior archistriatum
AId:	archistriatum (intermediate & dorsal)
AIv:	archistriatum (intermediate & ventral)
APH:	area perihippocampalis
E:	ectostriatum
FPL:	Fasciculus prosencephali lateralis
HA:	hyperstriatum accessorium
HD:	hyperstriatum dorsale
HIS:	hyperstriatum intercalatum supremum
HV:	hyperstriatum ventrale
Hp:	hippocampus
IMHV:	intermediate and medial hyperstriatum ventrale
LHV:	lateral hyperstriatum ventrale
LN:	lateral neostriatum
LPO:	lobus parolfactorius
MHV:	medial hyperstriatum ventrale
MN:	medial neostriatum
N:	neostriatum
PA:	paleostriatum augmentatum
PP:	paleostriatum primitivum
Se:	dorso-medial septum
TPO:	area temporo-parieto-occipitalis

General

Aa:	arachidonic acid
ANOVA:	analysis of variance
A _H :	high affinity component
A _L :	low affinity component
AST:	aristoloctic acid
B:	concentration of bound ligand or radioligand
B _{max} :	maximal binding of ligand or radioligand
Ca ²⁺ :	calcium ion(s)
cAMP:	cyclic adenosine monophosphate
cDNA:	complementary DNA
cGMP:	cyclic guanosine monophosphate
Cg-TX:	ω -conotoxin GVIA

Cl ⁻ :	chlorine ion(s)
cNMDA:	CPP preferring NMDA receptors
CNS:	central nervous system
CO:	carbon monoxide
D:	displacer
DAG:	diacylglycerol
2-DG:	2-deoxyglucose
dpm:	degradations per minute
EC ₅₀ :	apparent equilibrium dissociation constant
EPSP:	excitatory post synaptic potential
ES:	electro-shock
F:	concentration of free ligand or radioligand.
GluR1-7:	AMPA/KA glutamate receptor subunits (cloned)
GR33:	syntaxin
I:	inhibitor
ic:	intracerebral
IC ₅₀ :	apparent equilibrium inhibition constant
IEGs:	Immediate-early genes
IP ₃ :	inositol triphosphate
K ⁺ :	potassium ion(s)
KA1 & 2:	kainate receptor subunits (cloned)
K _D :	equilibrium dissociation constant
K _I :	equilibrium inhibition constant
L:	ligand or radioligand
LTD:	long term depression
LTP:	long term potentiation
MAP:	microtubule associated protein
MeA:	methylantranylate (or methylantranylate trained)
Mg ²⁺ :	magnesium ion(s)
mGluR:	metabotropic glutamate receptor
Na ⁺ :	sodium ion(s)
NCAM:	neural cell adhesion molecule
NDGA:	nordihydroguaiaretic acid
nH:	Hill coefficient
nNMDA:	NMDA preferring NMDA receptor
NO:	nitric oxide
NOS:	nitric oxide synthase
NR1 & 2:	NMDA receptor subunits (cloned)
NR2a-d:	NMDA receptor subunits (cloned)
NSB:	non-specific binding
PC:	Purkinje cell

PF:	parallel fibre
PKA:	protein kinase A
PKC:	protein kinase C
PLA ₂ :	phospholipase A ₂
PLC:	phospholipase C
rpm:	revolutions per minute
s.a:	specific activity
SCN ⁻ :	thiocyanate ion
SS:	steady state
TCB:	50mmol l ⁻¹ Tris-citrate buffer (pH 7.4)
VGCC:	voltage gated Ca ²⁺ channel
Y1:	first presentation of yellow test bead in visual discrimination test
Y2:	second presentation of yellow test bead in visual discrimination test
W:	water (or water trained)
Zn ²⁺ :	zinc ion(s)

Receptor agonists and antagonists

ACPC:	1-amino-cyclopropane carboxylic acid
<i>trans</i> ACPD:	<i>trans</i> :1-aminocyclopentyl-1,3-dicarboxylic acid
AMNH:	α -amino-3-[2-(3-hydroxy-5-methylisoxazole-4-yl)methyl-5-methyl-3-oxiisoxazoline
AMOA:	α -amino-3-[3-(carboxymethoxy-5-methylisoxazole-4-yl)propionic acid
AMPA:	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP3:	2-amino-3-phosphonopropionic acid
AP4:	2-amino-4-phosphonobutyric acid
BG10:	1,10-bis(guanidino)decane
5-BrW:	5-bromowillardine
CCG I:	(2S,3S,4S) α -(carboxycyclopropyl)glycine
CCG II:	(2S,3R,4S) α -(carboxycyclopropyl)glycine
CGP 39653:	DL(E)-2-amino-4-propyl-5-phosphono-3-pentanoic acid
CGS 19755:	<i>cis</i> -(\pm)-4-phosphonomethyl-2-piperidine carboxylic acid
CSA:	cysteine sulphinic acid
7-ClK:	7-chlorokynurenate
CNQX:	6-cyano-7-nitroquinoxaline-2,3-dione
CPP:	D-3-(2-carboxypiperazine-4-yl)-1-propyl-1-phosphonic acid
CPPene:	D(E) 3(2-carboxypiperidine-4-yl)-1-propenyl-1-phosphonic acid
CPPP:	<i>cis</i> -(\pm)-3-(2-carboxypiperidine-4-yl)propyl-1-phosphonic acid
D-AP5:	D-2-amino-5-phosphonopentanoic acid
D-AP7:	D-2-amino-7-phosphonopentanoic acid

DCS:	D-cycloserine
6,7-diCl-QX:	6,7-dichloroquinoxaline-2,3-dione
DTT:	dithiothreitol
DNQX:	6,7-dinitroquinoxaline-2,3-dione
GABA:	γ -aminobutyric acid
γ -LGLA:	γ -L-glutamyl-L-aspartate
GSSG:	oxidized glutathione
HA-966:	1-hydroxy-3-aminopyrrolidone-2
HCA:	homocysteate
KA:	kainate
LY 274614:	(\pm)-3SR,4aRS,6SR,8aSR-6-(phosphonomethyl)-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid
MCPG:	(RS)- α -methyl-4-carboxyphenylglycine
MK-801:	(+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclohepten-5,10-imine maleate
NBQX:	2,3-dihydroxy-6-nitro-7-sulfamyl-benzo(F)quinoxaline
NMDA:	N-methyl-D-aspartate
NPC 12626:	2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoic acid
PCP:	phenylcyclidine
PDA:	piperidine dicarboxylic acid
QA:	quisqualate
SKF 10047:	N-allylnormetazocine
TCA:	tricyclic antidepressant (eg, desmethylinipramine)
TCP:	N-[1-(2-thienyl)cyclohexyl]-piperidine
TG:	(tetrazole-5-yl)glycine
Z-cpr-AP4:	Z-cyclopropyl-2-amino-4-phosphonobutyric acid

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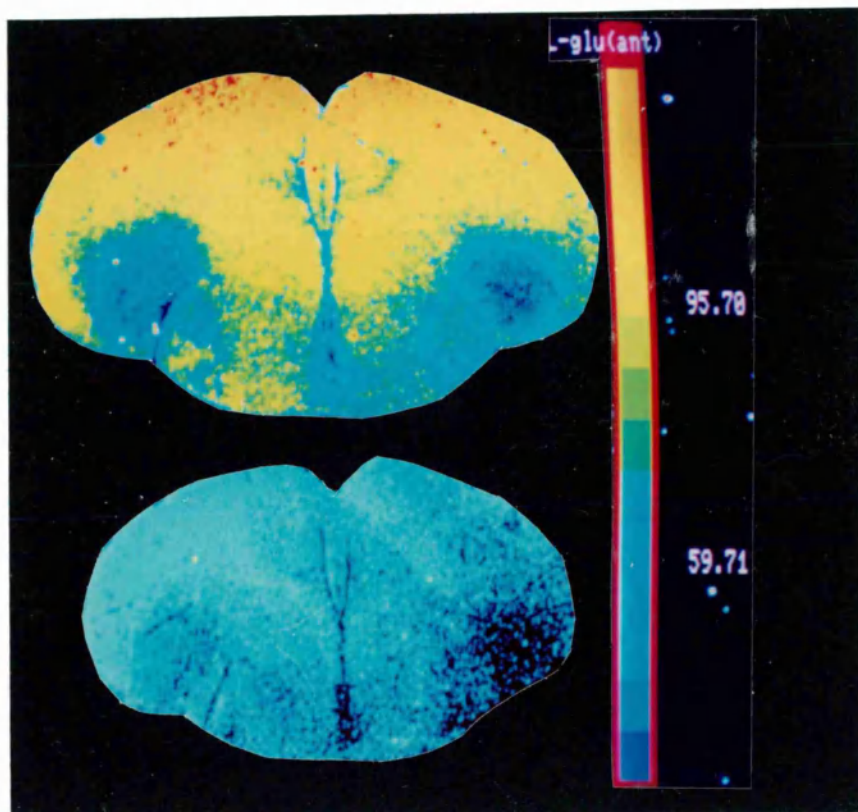
I also must thank those students and staff of the O.U, as well as various visitors, whose friendship and companionship has given me some of the best years of my life, and also of course for putting up with my “sense of humour”. Space precludes listing everyone, but in addition to those already mentioned above, I would particularly like to thank Jon Daisley, Milan Sojka, Adrian French, Lottie Hosie, John Owen, Fiona Freeman, Maria Gulinello, Tim and Fiza Doubell, Dave Fitzgerald, Lourdes Covarrubias, Jon Oliver, Ilana Hayer, Teré Valverde, Florentina Gonzales, Barbara Bruno, Johnny B, Phil McG and the DBs. I apologise to everyone I’ve missed out.

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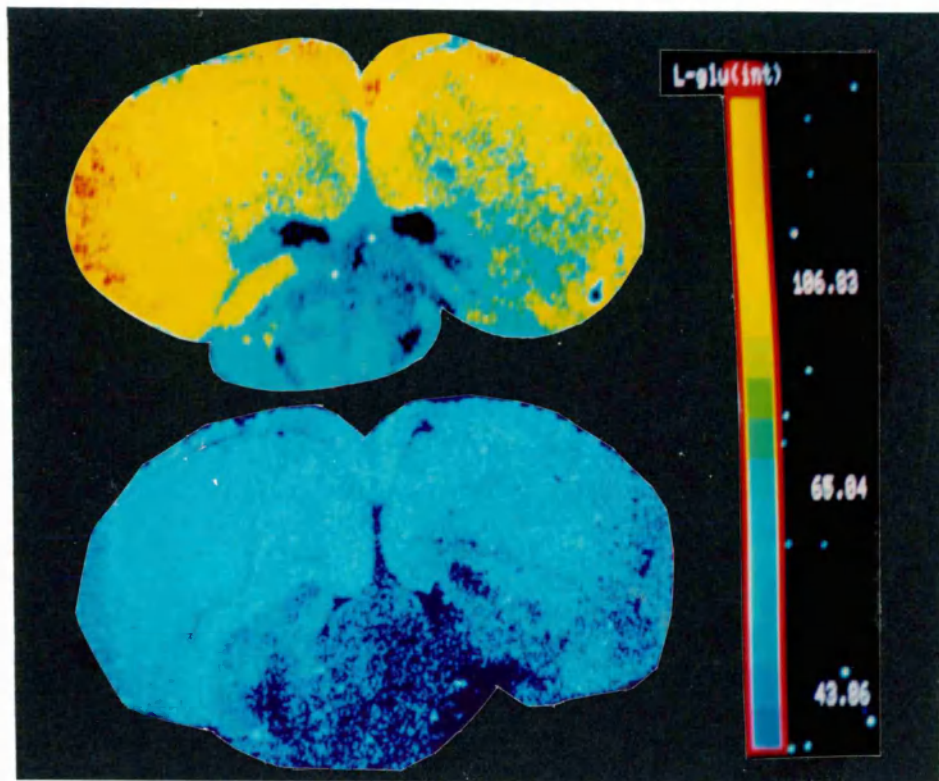
Colour plates



Colour plate 1: Joyce Loebel "Magiscan" image analysis set-up, used for the measurement of optical densities on autoradiograms developed for this thesis. On bench, from left to Right: Camera and light box, microscope (not used in the work described here), polaroid camera for slide making, image screen, key board, light pen for making measurements from images, PC (text) screen. Below bench: hard disc drives for "Magiscan system.



Colour plate 2: Pseudo-colour images taken from the screen of the “Magiscan” image analysis system, showing an autoradiogram of $[^3\text{H}]\text{L-glutamate}$ binding to a section from an anterior location of the chick forebrain, with a similar autoradiogram where $[^3\text{H}]\text{L-glutamate}$ was displaced by NMDA for non-specific binding.



Colour plate 3: Pseudo-colour images taken from the screen of the “Magiscan” image analysis system, showing an autoradiogram of $[^3\text{H}]\text{L-glutamate}$ binding to a section from an intermediate location of the chick forebrain, with a similar autoradiogram where $[^3\text{H}]\text{L-glutamate}$ was displaced by NMDA for non-specific binding.

Abstract

Memory formation depends on alterations in synaptic morphology, and specific L-glutamate receptor subtypes (NMDA and AMPA receptors) are known to play a key role in such synaptic plasticity. Changes in binding of neurotransmitters to discrete populations of receptors, in specific brain regions, could directly subserve memory related increases in synaptic efficacy. The cellular correlates of memory formation have been extensively studied in the chick brain, using one trial passive avoidance training. Alterations in receptor binding can be studied using quantitative autoradiography in conjunction with this paradigm. Also, this paradigm can be used to study the behavioural responses of chicks to drugs which act at specific receptors. These techniques were used to investigate the effect of passive avoidance on the binding characteristics of ionotropic L-glutamate receptors at specific times post-training, and of drug injections on the avoidance response. Pre-training intracerebral injection of NMDA receptor antagonists (MK-801, 7-ClK) were found to block the early stages of memory formation, whereas AMPA receptor antagonists did not. Injection of an AMPA receptor antagonist (CNQX) 4.5 or 5.5 h after training, however, blocked memory retention at 6.5 h. Autoradiographic data indicated the presence of two NMDA receptor subtypes in the chick forebrain, NMDA and CPP preferring receptors. The binding properties of these were found to be altered 30 min and 3 h after passive avoidance training. 30 min post-training, increased binding to NMDA receptors was found in the left IMHV and left LPO. At 3 h post-training increased numbers of nNMDA receptors were detected in the hippocampus and left AIv, whilst the affinity of cNMDA receptors increased in the AIv. The only alterations in the binding properties of AMPA receptors were at 6.5 h post-training, when increases in their affinity were found bilaterally in the IMHV and in the left PA (see abbreviations).

General introduction

During the latter half of this century interest in the mechanisms underlying memory and learning related synaptic plasticity, and more recently the role of L-glutamate receptors in these mechanisms, has burgeoned. This reflects among other factors, the increasing importance of developing new drugs with which to treat various disorders of the central nervous system (CNS)^{85,154,,217} and the development of powerful techniques which have rendered the various plastic changes associated with memory and learning increasingly accessible to investigation.

One such technique is *in vitro* quantitative receptor autoradiography. This can be used to determine the distribution and density of receptor types, and subtypes in various tissues *in situ*, in addition to allowing investigation of their pharmacology, and various processes in which they are involved^{24,138,204,212}.

L-glutamate receptors have been implicated in various aspects of memory and learning related synaptic plasticity, including augmentation and sensitization processes, which are involved in increasing synaptic efficacy^{205,212}. Quantitative autoradiography is ideal for the investigation of these processes.

A paradigm which has proved useful in demonstrating significant morphological, biochemical, and neurophysiological correlates of memory formation, is one trial passive avoidance training, in the domestic chick, *Gallus domesticus* (see section 4.0)^{177,202}. Here, this paradigm was used in quantitative autoradiographic and behavioural pharmacological investigations of the role of ionotropic L-glutamate receptors in memory formation. Before this research is discussed, however, details of the various types of L-glutamate receptors found in the vertebrate brain, their role in synaptic plasticity and learning and memory, and the various paradigms used to study this, will be reviewed (chapter 1) and the techniques and analysis used in the work discussed in this thesis will be described (chapter 2).

Chapter 1

L-glutamate receptors in learning and memory

1

Introduction

Since the 1960's there has been increasing interest in the role of amino acids, such as L-glutamate, as excitatory neurotransmitters²²². The receptors to which L-glutamate binds have been implicated in many physiological and neurophysiological processes involving synaptic plasticity^{7,10,21,54,71,141,212,227}. Recently, evidence has come to light that these receptors exhibit a functional and pharmacological multiplicity hitherto unknown in neuroscience¹⁸⁹. In section 2 of this review L-glutamate receptors are classified and this multiplicity is discussed. Sections 3 and 4 review the evidence for their role in memory and learning related synaptic plasticity and section 4 concentrates on L-glutamate receptors in the chick brain.

2

L-glutamate receptors in the vertebrate CNS

It is now generally accepted that L-glutamate is the major excitatory neurotransmitter in the vertebrate CNS^{47,155}. It has been estimated that it is the primary neurotransmitter at approximately 50% of synapses in the mammalian telencephalon (forebrain)¹²⁷. Other neurotransmitter candidates such as L-aspartate and quinolinate have now largely been excluded as possibilities for this role¹⁵⁵. It is possible that under some circumstances endogenous sulphur containing amino acids, such as L-homocysteate (L-HCA) or cysteine sulphinic acid (CSA), which are stored in, and presumably released from glia^{78,216}, can act at a specific receptor subtype (NMDA receptors)¹⁰⁴.

Like other neurotransmitters, L-glutamate is stored in vesicles within the pre-synaptic terminal and its release is dependent upon an influx of Ca^{2+} ions through voltage gated Ca^{2+} channels^{127,129}. L-glutamate concentrations can reach millimolar levels in the synaptic cleft, but this is controlled by two types of high affinity uptake system, one Na^+ dependent and the other Cl^- dependent¹³⁶.

L-glutamate can stimulate excitatory post-synaptic potentials (EPSPs) in nearly all neurons, and is also an important neurotransmitter in invertebrates, although in that sub-kingdom receptors for L-glutamate do not seem to correspond to any of the currently recognized subtypes found in vertebrates CNS^{26,47,127}.

L-glutamate binds to a number of functionally and pharmacologically distinct sub-populations of receptors in the vertebrate CNS. A primary distinction can be made between ionotropic (channel linked) and metabotropic (second messenger linked) receptor types. The nomenclature of ionotropic L-glutamate receptors is based upon the name of the selective agonist which has a higher potency at its corresponding receptor than at any other¹⁵⁶. Autoradiographic studies have shown that these selective agonists bind to anatomically distinct sub-populations of receptors, and recent evidence indicates that further distinct isoforms can be distinguished within these sub-populations^{136,189}. Furthermore, molecular isolation and cloning techniques, in addition to the development of new specific ligands, have revealed an even greater multiplicity^{116,143,189}. Metabotropic L-glutamate receptors (mGluRs) also comprise a family of subtypes based on differences in their structure, pharmacology and second messenger linkage. These were only recognized as a distinct family of receptors relatively recently, however, when molecular cloning revealed their diversity^{45,96,146,207}. (see table 1 for list of agonists and antagonists which bind to various L-glutamate receptors).

Table 1: Agonists and antagonists of L-glutamate receptors.

<u>Receptor subtype (binding site)</u>	<u>Selective agonists</u>	<u>Non-selective agonists</u>	<u>Selective antagonists</u>	<u>Non-selective antagonists</u>
NMDA (L-glutamate site)	NMDA L-HCA L-CSA quinolinate <i>cis</i> -ACPD <i>trans</i> -2,3-PDA L-CCG II D,L-TG	L-glutamate L-aspartate	D-AP5 D-AP7 CPP CPPene CPPP CGP 39653 CGP 55802A CGS 19755 NPC 12626 γ -LGLA LY 274614 LY 233536	
NMDA (glycine site)	D-serine DCS ACPC B6B21	glycine	HA-966 7-ClK	
NMDA (channel blockers)			MK-801 PCP TCP ketamine Mg^{2+}	Peptide YY
NMDA (Zn^{2+} site)			Zn^{2+} TCA BG10	
NMDA (σ -site)		Neuropeptide Y	SKF 10047	
NMDA (Polyamine site)	Spermine Spermidine			
NMDA (Redox site)	DTT		GSSG	
AMPA	AMPA ODAP	L-glutamate quisqualate D-HCA 5-BrW	NBQX 6,7-diCl-QX cyclothiazide CNQX	DNQX
metabotropic	<i>trans</i> -ACPD L-CCG-I	L-glutamate quisqualate D-HCA 5-BrW	AP3 AP4 MCPG	
kainate	kainate domoate	L-glutamate quisqualate D-HCA 5-BrW	AMOA AMNH	CNQX DNQX
L-AP4	L-AP4 Z-cpr-AP4	L-glutamate		

List of references: 11, 15, 34, 52, 67, 66, 76, 80, 82, 87, 102, 103, 106, 110, 116, 124, 131, 143, 150, 154, 162, 168, 171, 185, 186, 206, 210, 221, 222, 223, 230.

2.1

Ionotropic L-glutamate receptors

Ionotropic L-glutamate receptors are conventionally divided into two major classes: NMDA receptors and non-NMDA receptors. NMDA receptors are so called because they are selectively activated by the glutamate analogue, N-methyl-D-aspartate (NMDA). Non-NMDA receptors are classified according to their preferential responses to a number of synthetic alkaloids¹²⁷. In fact, at least three major subclasses of ionotropic receptors (the NMDA subtype, plus two distinct non-NMDA subtypes) can be distinguished in the vertebrate CNS. This is based, largely on pharmacological, neurophysiological, and quantitative autoradiographic evidence^{136,138,189} but also, more recently, on structural analysis^{91,92} as well as molecular isolation and cloning techniques^{140,189}.

2.1.1: *Non-NMDA receptors*

Non-NMDA L-glutamate receptors were first characterised using the selective agonists kainate(KA) and quisqualate(QA). It has since become clear, however, that the subpopulation of receptors to which QA binds is not homogeneous and that, in fact, it binds to metabotropic receptors in addition to ionotropic non-NMDA receptors^{127,222}. The alkaloid non-NMDA receptor agonists, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid (KA), bind to distinct receptor populations, although there is a degree of overlap in their binding (see below). Thiocyanate (SCN^-) ions enhance AMPA but not KA binding⁷², and AMPA and KA receptors also exhibit different rank orders of potency for other agonists and antagonists²³⁰. KA receptors are selectively inhibited by the antagonists AMOA and AMNH⁶⁶. AMPA receptors are selectively inhibited by the competitive antagonists NBQX and 6,7-diCl-QX²²³, and by the non-competitive antagonist cyclothiazide which lowers the receptor's affinity for AMPA⁸⁰. CNQX and DNQX competitively inhibit both AMPA and KA receptors (AMPA > KA)^{66,82,223}.

The autoradiographic distributions of [^3H]AMPA and [^3H]KA do not coincide. In general, relatively high densities of AMPA receptors occur in most brain regions^{84,136}. KA

receptors have a more localized binding distribution and are particularly dense in the cerebellum (granule layer in mammals, molecular layer in birds)^{84,137}. AMPA and KA receptors both exhibit low and high affinity binding components^{72,230}. In addition to its own high affinity receptors, KA binds to AMPA receptors with a lower affinity than AMPA, and low affinity AMPA receptors have been considered by some, to be unitary AMPA/KA receptors¹⁵⁸.

AMPA receptors are the most abundant class of excitatory ionotropic receptor in the vertebrate brain^{72,165} and they mediate the majority of all fast excitatory neurotransmission¹⁸⁹. On most neurons in the mammalian brain AMPA receptors are permeable to Na⁺ and K⁺, but have low Ca²⁺ permeability. Neurophysiological responses to AMPA have two component kinetics, a fast desensitizing (“peak”) component, and a steady state (SS) component. KA activation of AMPA receptors is non-desensitizing, ie, current persists in the continued presence of agonist, whereas high affinity KA receptors mediate fast desensitizing current¹⁸⁹. Neurophysiological studies of single channel responses using outside-out patch clamping techniques, have distinguished three different KA sensitive receptors, as well as a KA insensitive high affinity AMPA receptor¹⁶⁴. Also, pharmacological studies have revealed high and low affinity components of [³H]AMPA binding^{72,80}. These indications of multiplicity complement the findings of molecular cloning studies.

Nine different ionotropic non-NMDA receptor subunits have been cloned from rat brain cDNAs^{64,189}. AMPA receptors comprise the subunits GluR1 - GluR4 which are approximately 900 amino acids in length. They form functional L-glutamate sensitive ion channels *in vitro* when expressed alone, or when co-expressed in pairs. The DNA sequences of GluRs 1-4 show that they are closely related (69-74% homology)^{64,196}. Each of these subunits can occur as one of two splice variants, “flip” or “flop”, which are alternately spliced with respect to an exonic sequence of 38 residues. In pre-natal brain only “flip” subunits are expressed, and these form receptors which desensitize more slowly than adult receptors containing both the “flip” and “flop” forms. The presence of the “flop” variant increases the ratio between the “peak” and SS components of AMPA receptor mediated

current. In receptors containing only the "flopp" form the SS component is virtually absent¹⁸⁹. The property of low Ca^{2+} permeability depends on the presence of the GluR2 subunit. Channels which comprise any combination of GluR1, 3 or 4 are Ca^{2+} permeable. GluR2 is present in most mammalian neuronal AMPA receptors. Ca^{2+} permeable non-NMDA receptors, however, have been revealed in rat hippocampal CA3 synapses¹⁵¹. Judging by GluR subunit distribution¹⁹⁶ these are likely to be GluR1/GluR3 AMPA receptors. Bergman glia also contain Ca^{2+} permeable (GluR1/GluR4) AMPA receptors¹⁸⁹. In birds the situation seems to be rather different. Here, a high proportion of AMPA receptors on forebrain neurons are Ca^{2+} permeable²⁷.

KA receptors can be generated *in vitro* from GluR5, 6, and 7 and KA1 and 2. The expression of KA1, KA2 or GluR7 alone does not result in functional channels, but when any one of these is combined with either GluR5 or GluR6 the resulting receptor has properties typical of high affinity KA receptors. Also, the combined expression patterns of these five subunits is similar to the autoradiographic distribution of KA receptors^{137,189}. GluR's 5 and 6 are closely related to each other (80% homology) but are more distantly related to GluR's 1-4 (<40% homology)⁶⁴.

A number of studies of chick, frog (*Rana*) and goldfish brain have indicated the existence of high affinity KA binding proteins which are highly insensitive to both AMPA and L-glutamate. These are found almost exclusively in cerebellar glia. Cloning studies show that they are not very closely related to mammalian KA receptors and it is not clear whether they are, in fact, KA receptor subunits^{58,79,83,197}. A number of L-glutamate receptor subunits purified from *Xenopus* brain have been shown to be interchangeable between different receptor types. They can form NMDA receptors, unitary AMPA/KA receptors or unitary AMPA/KA/NMDA receptors, depending on the combination of subunits comprising the receptor⁸¹. Such evidence seems to indicate that L-glutamate receptors, among vertebrate species, vary to a much greater extent than was previously suspected.

2.1.2: NMDA receptors

The NMDA receptor is the best characterized of all L-glutamate receptor subtypes. A number of highly specific antagonists of the NMDA receptor (both competitive and non-competitive - see table 1) have been developed, largely owing to its important clinical role^{85,217,227}. These have revealed the complexity of the pharmacology and regulation of the NMDA receptor, in addition to the existence of a number of subtypes^{124,136,143,154,206}, and have allowed detailed investigation of its various physiological roles²²². The molecular cloning of various NMDA receptor subunits has further added to understanding of this receptor and its subtypes^{140,189}.

NMDA receptors are physiologically and pharmacologically very different from the other types of ionotropic L-glutamate receptor, already discussed. In contrast to relatively simple ligand gated channels such as AMPA and KA receptors, NMDA receptors are combined ligand and voltage gated channels. Their activation requires both post-synaptic depolarization and ligand binding^{11,222} which are regulated, interactively, by various allosteric and channel blocking binding sites^{124,154}. NMDA receptors mediate a low amplitude, slow activating and slow decaying current¹²⁴ and are highly permeable to Ca^{2+} , in addition to the major current carriers Na^+ and K^+ ¹¹. The voltage dependency of NMDA receptor activation results from blockage by Mg^{2+} , which binds to a site deep within the channel¹²⁴ and dissociates when post-synaptic depolarization reaches a threshold level (maximum block occurs at -80mV)^{11,110}. Opening of the NMDA receptor channel also requires binding of two co-agonist neurotransmitters, because in addition to L-glutamate, glycine is also required. Each of these ligands allosterically potentiates the binding of the other, ie, they mutually increase their affinities for the receptor^{117,124}. The efficacy of NMDA receptor mediated responses in the presence of either agonist alone, is much lower than that in the presence of both agonists¹¹⁷.

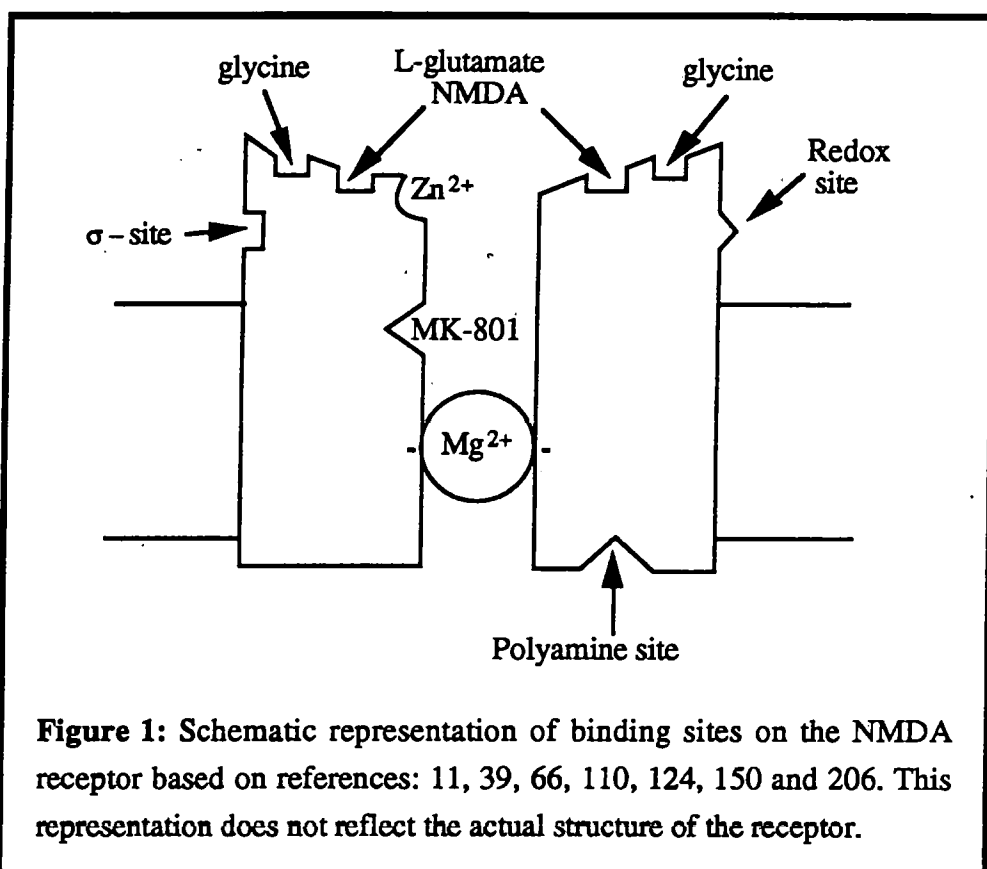
Evidence from studies involving activation kinetics, suggests that L-glutamate and glycine each have two binding sites on the NMDA receptor³⁹. Selective antagonists at the L-

glutamate site include D-2-amino-5-phosphonopentanoic acid (D-AP5), its 7-phosphono isomer D-AP7 and 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP)¹⁵⁴, as well as newer more potent, and more selective antagonists such as CGP 55802A¹¹⁶ and CGP 39653¹⁴³. Also, a number of specific new and potent L-glutamate site agonists, derived from glycine, have recently been developed, such as (2S,3R,4S)- α -carboxycyclopropyl glycine (L-CCG-III) and D,L-(tetrazole-5-yl) glycine (D,L-TG)^{52,106,186}. Selective glycine site antagonists include HA-966, which allosterically inhibits L-glutamate binding, and 7-chlorokynurenate (7-ClK) which is a pure competitive antagonist¹⁰³. Selective glycine agonists include 1-amino-cyclopropane carboxylic acid (ACPC)¹²⁴ and the partial agonist D-cycloserine (DCS)⁶⁷.

Non-competitive antagonists have become important tools for investigating the basic mechanisms of the ion channel of the NMDA receptor. Compounds such as phenylcyclidine (PCP) and, more specifically and potently, MK-801, block NMDA mediated responses by binding in a use dependent manner (ie, antagonist binding increases as the agonist concentration increases) to a site within the ion channel itself^{11,48,110}. Identification of endogenous ligands at this site, has not been so clear cut, but there is evidence that peptide YY does bind to this site, and could be an endogenous channel blocker¹⁷¹.

NMDA receptors have a number of other regulatory binding sites. These include a site which binds polyamines, such as spermine and spermidine. This site potentiates L-glutamate and glycine binding, while it is, itself, differentially modulated by these¹⁵⁰. It is also thought to be cooperative with a Zn^{2+} binding site, situated close to the channel, which binds 1,10-bis(guanidino)decane (BG10) and tricyclic antidepressants as selective antagonists^{11,168}. The Zn^{2+} binding site is thought to be important in the modulation of NMDA receptor mediated PKC translocation¹². There is also a σ -opiate site, outside the channel, on the NMDA receptor, which binds the antagonist SKF10047. Although the functionality of this site is still obscure, it is thought to bind neuropeptide Y as an endogenous ligand^{102,110,171}. Another site seems to regulate the NMDA receptor via a redox mechanism. Reducing agents, such as dithiothreitol (DTT), acting at this redox site,

potentiate NMDA receptor mediated responses, whereas oxidizing agents such as oxidized glutathione (GSSG) inhibit such responses^{76,206}. (See figure 1 for summary of binding sites).



At least four pharmacologically distinct NMDA receptor subtypes have been identified in rat brain^{34,136}. These differ in their binding affinities for agonists and antagonists, are differentially modulated by glycine and polyamines, and differ in their regional distributions^{34,136,143}. In the forebrain two major NMDA receptor populations (nNMDA and cNMDA) can be easily distinguished. One (nNMDA) displays a higher affinity for the agonists L-glutamate and NMDA than for competitive antagonists, such as CPP, and the other (cNMDA) has higher affinity for CPP and related antagonists than for agonists^{34,136}. The autoradiographic binding distribution of [³H]CPP in rat brain is very different to that of NMDA sensitive [³H]L-glutamate. Also, 'cold', CPP and NMDA display a complementary ability to displace [³H]L-glutamate binding^{34,136}. NMDA sensitive [³H]L-glutamate binding is relatively very dense in the striatum and dorso-lateral septum, whereas

[³H]CPP binding density is relatively high in the lateral thalamus. The distributions of nNMDA and cNMDA receptors also differ to some extent in other regions (see ref 34 for full review). L-glutamate has a higher affinity and potency at nNMDA receptors than at cNMDA receptors¹³⁵, and in addition their binding affinities are differentially modulated by glycine¹⁴³. The antagonists [³H]CPP and [³H]CGS 19755 exhibit two distinct components of binding in rat cortical membranes, high affinity to cNMDA receptors and low affinity to nNMDA receptors. Binding of a more specific antagonist, [³H]CGP 39653, has a single high affinity component which correlates to the high affinity component of CPP binding¹⁴³. [³H]CGP 39653 binding, however, is allosterically displaced by glycine in a biphasic manner, suggesting that cNMDA receptors could comprise further subpopulations. A third NMDA receptor subpopulation, which seems to be exclusively cerebellar in mammals, has a very low affinity for MK-801 and other channel blocking antagonists as well as CPPene and Homoquinolinate (HQ)^{34,135,136}. A fourth, which is almost exclusively thalamic with its highest levels in the medial thalamic nuclei, is also relatively insensitive to CPPene and HQ, but has a relatively high affinity for the antagonist LY 233536³⁴.

Two groups of NMDA receptor subunits have been cloned. These include seven splice variants of the NR1 subunit (NR1a-g) and four different NR2 subunits (NR2A-D)³⁴. Functional NMDA receptors can be reconstituted as heteromeric structures by co-expressing NR1 with any one NR2 subunit. NR2 subunits do not form functional channels except in combination with NR1¹⁸⁹. The pharmacological and physiological properties of such reconstituted NMDA receptors, including L-glutamate and glycine sensitivity, strength of Mg²⁺ block, deactivation kinetics and single channel characteristics, vary according to subunit composition^{140,147,189}

The distributions of NR1 and 2 mRNAs have been determined in rat brain using *in situ* hybridization. NR1 is expressed throughout the brain, although its NR1b splice variant is restricted to the cerebellum^{140,147}. NR2A expression is widespread in the forebrain and cerebellum, but is notably low in the striatum and lateral-septum. NR2B expression is confined to the forebrain, including the thalamus and striatum. NR2C is confined to the

cerebellum and NR2D mRNA is expressed mainly in the thalamus^{34,140}. These anatomical distributions have been correlated with the autoradiographic distributions and pharmacologic properties of cNMDA (NR1/NR2A), nNMDA (NR1/NR2B), cerebellar (NR1/NR2C) and medial thalamic (NR1/NR2D) NMDA receptors³⁴. The properties of cNMDA receptors have been shown to depend on the presence of the NR2A subunit and an NR1 splice variant with insert 1 present (NR1+), and those of nNMDA receptors have been shown to depend on the presence of NR2B and an NR1 splice variant lacking insert 1 (NR1-). The anatomical distributions of NR1+ and NR1- splice variants have also been shown to correlate reasonably well with those of cNMDA and nNMDA receptors respectively³⁴. Apart from NR1b (cerebellar) other NR1 splice variants have anatomical distributions which do not correspond to any of the four apparent NMDA receptor subtypes and do not confer significant differences in L-glutamate site pharmacology³⁴, although it is possible that their presence may result in other differential properties.

The existence of multiple NMDA receptor isoforms may have important functional implications, regarding the differing roles that they might play in synaptic plasticity, and there is, already, some evidence to indicate this^{60,122} (see section 4.3).

A recently cloned pre-synaptic protein, GR33 or syntaxin, when reconstituted in *Xenopus* oocytes, has been found to form functional L-glutamate gated Ca^{2+} permeable channels with pharmacology similar to that of NMDA receptors, but with different channel properties. GR33 is believed to be linked to N-type voltage gated Ca^{2+} channels, forming pre-synaptic NMDA receptors which may influence neurotransmitter release¹⁹⁴.

2.2

Metabotropic and L-AP4 receptors

Metabotropic L-glutamate receptors were, until recently, believed to comprise a single, discrete receptor population, and to exert their action through phosphoinositide hydrolysis, via a pertussis toxin sensitive G-protein and phospholipase C (PLC) activation,

resulting in intracellular Ca^{2+} mobilization^{15,45,156}. This, however is only one of a family of metabotropic L-glutamate receptors^{146,207}. The 1S,3R enantiomer of the L-glutamate analogue *trans*- 1-amino-cyclopentyl-1,3-dicarboxylic acid (*trans* ACPD) and (2S,3S,4S)- α -(carboxypropyl)glycine (L-CCG-I) are selective agonists, while 2-amino-3-phosphonopropionic acid (AP3) and its 4-phosphono isomer AP4 are selective antagonists^{15,230}. (RS)- α -methyl-4-carboxyphenylglycine (MCPG) is a specific mGluR antagonist⁸⁷.

Six distinct metabotropic L-glutamate receptor subtypes, mGluR1-6 have been cloned^{123,207}. These share no sequence similarity with other second messenger linked receptors, but have a relatively large degree of sequence similarity with GluR's 1-6^{123,207}. The properties of these subtypes differ not only in the rank order of agonist potencies and distribution in the brain, but also in the second messenger systems to which they are linked. mGluRs 1, and 5 are linked to phosphoinositide hydrolysis, as described above, mGluRs 2, 3, 4 and 6, however, are linked to adenylate cyclase and act by inhibiting cyclic AMP formation²⁰⁷. This latter form of signal transduction may be important in processes where mGluRs and AMPA receptors interact, as cAMP dependent protein kinase A (PKA) has been shown to regulate the Ca^{2+} permeability of GluR1/GluR3 channels¹⁰⁰. The six mGluRs can be further classified into subfamilies according to sequence similarities and properties, as follows: mGluR1/mGluR5, mGluR2/mGluR3, mGluR4/mGluR6¹⁴⁶. One other metabotropic L-glutamate receptor which has not been cloned, but is distinguishable from the others pharmacologically, has similar signal transduction properties to the cAMP linked mGluRs, but has similar pharmacology to the L-glutamate binding site of the NMDA receptor⁹⁶.

Another non-NMDA receptor type has been identified in certain excitatory pathways, from the specific depressant effect of the L stereo-isomer of AP4 on L-glutamate induced responses, in the absence of antagonist effects²²⁴. It is possible that L-AP4 acts as an agonist at ionotropic, negative feedback, L-glutamate autoreceptors, which are distinct from AMPA or kainate receptors²²². L-AP4 receptors are difficult to characterise autoradiographically, as they are freeze labile, and also because L-AP4 does not seem to bind to its receptors, in the

absence of Cl^- ions¹³⁹. The L-AP4 receptor has been distinguished from other non-NMDA receptors, in that it mimics L-glutamate action in hyperpolarizing certain retinal cells¹⁶². Recently doubt has been thrown upon this by the characterization of mGluR6 which effects a similar response. L-AP4 receptors, however, do not bind quisqualate³⁷, and in addition, a number of conformationally constrained L-AP4 analogues have recently been developed which have allowed further characterisation of the receptor. The most potent of these is Z-cyclopropyl-AP4 (Z-cpr-AP4)¹⁶².

3

Learning, memory and glutamate receptors

Although everyone intuitively understands what is meant by the terms learning, and memory, they have been notoriously difficult to define formally. Perhaps the earliest attempt to do this was by the Italian psychiatrist Tanzi (1909)²⁰⁸ who defined memory as “the capacity that the states of consciousness have of renewal in the form of recollections or reminiscences through the agency of the associative processes”. More recently, Rose (1992)¹⁷⁶, has defined learning as “a response by an animal to a novel situation such that, when confronted subsequently with a comparable situation, the animals behaviour is reliably modified in such a way as to make its response more appropriate (i.e. adaptive)”; recall as “the expression of the modified behavioural response at some time subsequent to the initial learning”; and memory as “some type of record inside the organism, by which the information acquired during the learning is stored in such a form that it can be made available to modify subsequent behaviour”.

Many other definitions of these concepts could be quoted, but those of Rose are very typical and cover the main points made by most. Most definitions agree that learning and memory are experience dependent and can only be inferred from observation of how an organism's behaviour is modified as a result of its experiences. Another important point which is common to most definitions is reliability. A behavioural modification resulting from learning in an individual must be seen to occur repeatedly, or must occur similarly in each

member of a group. The reference to memory storage in the last of the above quotations of Rose, as well as the use of the word “adaptive” in the first, implies another point which is common to most definitions of memory, i.e, that it must be long-lasting. It is generally agreed that memory is initially acquired as a transient phenomenon (short term memory), which can be consolidated as long term memory if the behavioural modification is important enough to the organism as an adaptation^{61,159,165,176,190}. Squire (1987)¹⁹⁹ defines short term memory as referring to “a system that retains information only temporarily in a special status while it becomes incorporated, or transfers, into a more stable, potentially long term store”.

Many authors also define two main categories of learning. These are associative learning, where an organism learns to associate one stimulus with another, and non-associative learning which includes phenomena such as habituation and sensitization⁶¹. This thesis will mainly be concerned with the former. Associative learning is, sometimes, also divided into more than one category. The most commonly used of these are classical and instrumental conditioning⁶¹. In classical, or Pavlovian, conditioning an “unconditioned stimulus”, which evokes a particular behavioural response, is associated with a neutral “conditioned stimulus”, which will eventually evoke the same response on its own as a result of the association. In instrumental conditioning the frequency or intensity of a particular spontaneous behavioural response is altered by a reinforcing stimulus.

Two further subdivisions have been defined, with reference to human memory¹⁹⁹. These are “declarative” and “procedural” memory. Declarative memory is “memory for facts and episodes”, ie, it involves data and facts (eg specific concepts, vocabulary or time and place events) that have been acquired through learning and can be consciously recalled. Procedural memory is “memory for skills and other cognitive operations”. It is not accessible to conscious recall, but is expressed as learned skills or “modifiable cognitive operations” (eg, riding a bicycle, reading or understanding a language). Declarative memory is also sub-divided into “episodic” memory, which concerns temporally dated events, and semantic memory which concerns specific facts and concepts. As a result of studies involving human amnesic patients, declarative and procedural memory are considered to

differ not only in the type of information that is stored and how it is used, but also in the neuronal systems that are required for this storage. Classical conditioning is considered to be a type of procedural memory¹⁹⁹.

The distinction between short and long term memory has been amongst the most useful concepts in the study of the cellular correlates of memory formation. Short and long term memory are generally seen as separate processes which are initiated simultaneously, but whereas short term memory decays over a period of minutes to hours, long term memory becomes permanent if it has developed sufficiently by the time short term memory has decayed^{128,173}. Not only is short term memory much more easily disrupted than long term memory, as has been shown in human amnesic patients¹⁹⁹, but it can also be disrupted electrically, eg, administration of transcranial subconvulsive electroshock to domestic chicks less than 5 min after passive avoidance training (see section 4.1.2) results in amnesia¹⁸⁰. This lability of short term memory suggests that it is stored electrically in some way, perhaps through altered activity within neural networks in the brain¹⁷³. This type of electrically stored memory trace has also been called “working memory” which is defined as a “memory buffer in which to maintain information while it is being processed¹⁹⁹. Many authors, however, use the term “short term memory” in reference to memory associated changes in synaptic biochemistry or structure which are initiated at the time of learning and are quickly completed¹⁹⁹. In McGaugh’s multiple trace hypothesis¹²⁸ four separate stages of memory formation are defined in the form of memory traces with successively later peaks and longer rates of decay. The electrically coded trace, which peaks earliest and decays most rapidly, is called “buffer” and this is followed by short term, intermediate term and long term memory traces.

The distinction between short and long term memory has been made using many animal models, from relatively primitive invertebrates to mammals. Perhaps the best known invertebrate model of learning is sensitization of the gill and syphon-withdrawal response in the mollusc *Aplysia californica*, where delivery of a noxious stimulus enhances the response to a test stimulus. This has been shown to have a short term stage, which is dependent upon

voltage gated Ca^{2+} channels and can be modulated by serotonin and cAMP^{13,36}, and a long term stage which depends upon protein synthesis³⁶. Invertebrate associative learning models include the use of a classical conditioning olfactory discrimination task involving various mutants of the fruit-fly *Drosophila melanogaster*^{30,62} use of which has lead to the elucidation of a multi-trace learning model remarkably similar to that proposed by McGaugh (1968)^{30,128}.

The most useful model for study of the temporal aspects of learning and memory in birds has proved to be passive avoidance training of young domestic chicks (*Gallus domesticus*)³⁸ (see section 4.1.2). Gibbs and Ng⁶⁹ distinguished between three stages of memory formation: short term, intermediate term and long term, for a colour discrimination passive avoidance task, using pharmacological manipulations. These stages have since been further elucidated by Rosenzweig et al¹⁸¹. Rose and co-workers have also accumulated a body of evidence, using passive avoidance training, for at least two stages of memory formation¹⁷⁷ (see section 4.3).

Many and various paradigms have been used to study learning and memory in mammals (some of which are mentioned in section 3.2), although these have mainly concentrated on the distinction between acquisition and long term retention of memory. Acquisition and retention stages of spatial memory, for example, have been elucidated using an eight arm radial maze task for rats^{191,215}, and a number of studies which have used a classical conditioning task for rabbits have similarly distinguished between these stages^{19,97,210}. Perhaps the most useful distinctions between short and long term memory in mammals, however, come from studies of human amnesic patients. It was in this context that these stages of memory and their properties were first defined (see Squire, 1987¹⁹⁹, Chapt 10 for review).

It is generally held that storage of long term memory involves alterations in synaptic structure and the connectivity of neuronal networks^{166,177}. The most popular model for such synaptic remodelling has been that postulated by D.O.Hebb in the 1940s.

Hebb's postulate of learning states that³³

"When an axon of cell A is near enough to excite cell B, or repeatedly, or consistently, takes part in firing it, some growth process or metabolic change takes place in one or both cells, such that A's efficiency, as one of the cells firing B, is increased."

A Hebbian synapse is defined as one that uses a "time dependent, highly local and strongly interactive mechanism to increase synaptic efficacy, as a function of the conjunction or correlation between pre and post-synaptic activity³³".

3.1

L-glutamate receptors in LTP

One neurophysiological phenomenon involving synaptic plasticity, which displays typical Hebbian features and is thought by many to be a reasonable model for the processes involved in learning and memory, is long term potentiation (LTP) which has been defined as "activity-dependent potentiation of synaptic efficiency"^{21,33}. NMDA, AMPA and metabotropic L-glutamate receptors are involved in the induction and maintenance of various types of LTP^{5,17,21,45,121}. These are divided into two broad categories, NMDA receptor-dependent and independent LTP. NMDA receptor-dependent LTP includes associative LTP, E-S potentiation and non Hebbian LTP. NMDA receptor independent LTP includes paired-pulse facilitation, post-tetanic potentiation (PTP) and mossy fibre LTP^{21,33}. Another related phenomenon is long term depression (LTD)³¹.

3.1.1: NMDA receptor-dependent LTP.

Associative LTP has been most extensively studied in the Schaffer collateral/commissural inputs to the CA1 pathway, as well as in the perforant path to the dentate gyrus, in the mammalian hippocampus^{21,43} but has also been shown to occur in the neo-cortex¹⁶⁶. Brief, repetitive, high frequency stimuli (tetani) result in the long lasting increase in synaptic efficacy defined as LTP^{21,33}. The earliest observations of this

phenomenon were described by Bliss and Lomo in the late 1960s and early 1970s²³ in rabbit hippocampus, although most subsequent work has been done using that of the rat. The onset of associative LTP occurs within a few seconds and in the intact animal it can last for several weeks. Its induction depends upon increases in post-synaptic Ca^{2+} concentration mediated, initially, by NMDA receptors (NMDA receptor antagonists such as D-AP5, MK-801 and 7-ClK block LTP induction). This elevation of post-synaptic Ca^{2+} triggers a cascade of pre and post-synaptic biochemical processes, culminating in changes in synaptic morphology and a sustained increase in post-synaptic bursting (ie, the rate of action potential generation)^{21,121}.

The associativity of LTP induction is imparted by NMDA receptors, which function in an analogous manner to logical AND gates in computers due to their dual voltage and ligand gated mode of activation³³. The post-synaptic depolarization necessary to relieve the NMDA receptor's voltage sensitive Mg^{2+} block is usually achieved via AMPA receptor mediated EPSPs^{21,121,144}. Also, occupation of both the L-glutamate and glycine sites on NMDA receptors is essential for LTP induction^{21,121,153}.

Pre-synaptic changes associated with LTP, including increased L-glutamate release, may be initiated and/or maintained by a number of signaling systems, including the retrograde messengers nitric oxide (NO), arachidonic acid (Aa) and carbon monoxide (CO)^{21,25,44,142,161,233}. Also presynaptic NMDA receptors may be involved. A transient increase in the amount of mRNA coding for GR33 is associated with LTP induction^{194,195}.

AMPA receptors mediate low frequency, fast synaptic, transmission before and after the induction of LTP. Increases in this type of transmission are associated with the maintenance of LTP in its later stages and result, at least in part, from increased AMPA receptor affinity^{21,121,213}. Although NMDA receptors are not involved in the maintenance of hippocampal LTP, some evidence indicates that they are involved in the later stages of neocortical LTP¹⁶⁶. Metabotropic L-glutamate receptors are also involved in both the induction of associative LTP, and in its later stages. Selective activation of phosphoinositide linked mGluRs potentiate NMDA receptor mediated currents via activation of protein kinase C (PKC), which phosphorylates specific sites on NMDA receptors reducing their Mg^{2+}

channel block^{5,17}. Also, the mGluR antagonists AP3 and AP4 reduce the duration of LTP, ie, they block its maintenance²¹. LTP maintenance may be modulated by adenylate cyclase linked mGluRs. cAMP dependent PKA phosphorylates AMPA receptors, increasing their affinity and, thus, potentiating AMPA receptor mediated responses¹⁰⁰.

E-S potentiation and non-Hebbian LTP are non-specific types of NMDA receptor dependent LTP. Non-Hebbian LTP occurs not only at synapses where pre and post-synaptic activity are correlated, but also extends to synapses at neighbouring cells, whether or not they are active²¹.

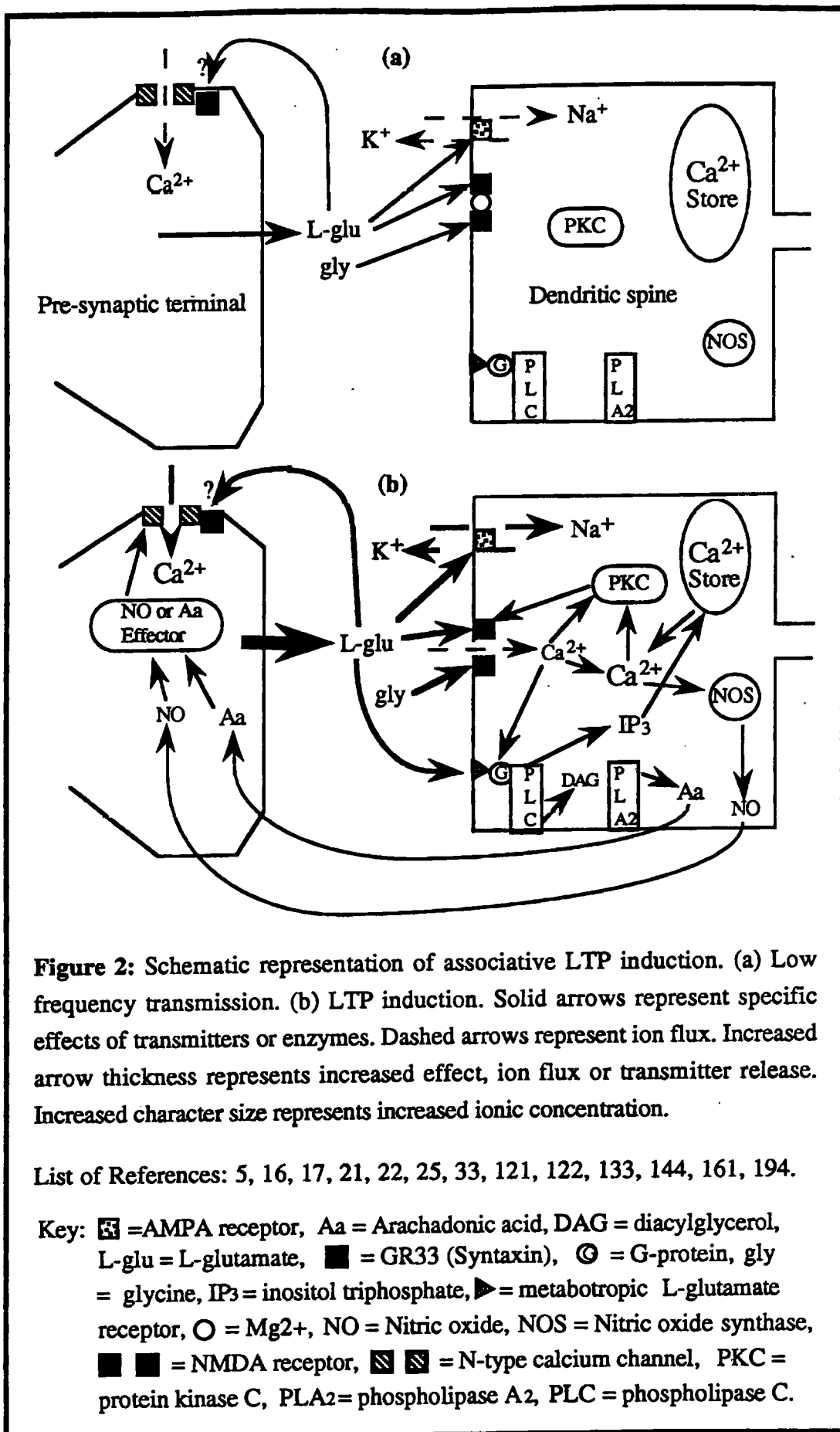
3.1.2: NMDA receptor-independent LTP.

Of the three types of NMDA receptor independent LTP, mossy fibre LTP is probably the most important. So called "mossy fibres" terminate in the CA3 subfield of the mammalian hippocampus, a region in which there are very few, if any, NMDA receptors. Mossy fibre LTP is slow onset and non-associative, and is not blocked by NMDA receptor antagonists. It is, however, induced by the selective metabotropic L-glutamate receptor agonist *trans* - ACPD, and AP3 has been shown to block mossy fibre LTP in hippocampal slices. This evidence indicates that mossy fibre LTP is induced by intracellular Ca²⁺ mobilization via phosphoinositide linked mGluRs^{21,45,136,230}, although Ca²⁺ permeable AMPA receptors have also been implicated^{45,100}.

Other types of NMDA receptor independent LTP include paired pulse facilitation and PTP. These are both general features of excitatory synaptic transmission and, under the usual conditions of LTP induction, have a duration of a few minutes at most²¹.

3.1.3: Long term depression.

LTD is a model of synaptic plasticity which has been found to occur in the mammalian cerebellum and has been implicated in cerebellar motor learning. Long lasting



depression of cerebellar parallel fibre (PF) - Purkinje cell (PC) transmission occurs after conjugative stimulation of climbing and parallel fibres. Ca^{2+} influx through voltage gated calcium channels is the initial step in LTD induction at PF-PC synapses, along with the activation of mGluRs⁵⁵. Activation of PC AMPA receptors also seems to be required, and NO has a role to play. NO synthase inhibitors block cerebellar LTD^{31,55,88}. There is some controversy over whether LTD occurs in other brain regions, particularly the hippocampus^{9,160}.

3.2

L-glutamate receptors in mammalian learning and memory

In addition to postulated similarities between the plastic processes underlying LTP and those underlying the acquisition and retention of memory, there is also more direct evidence implicating L-glutamate receptors in learning and memory.

Learning and recall are impaired by treatment with selective NMDA receptor antagonists^{41,131,198,210}. Spatial learning of rats in a Morris water maze is inhibited after treatment of the hippocampus with D-AP5^{141,142}, and MK-801 has been found to impair the acquisition of spatial working memory in an eight arm radial maze task, but not the retention of such a task in a familiar environment¹⁹¹. D-AP5 impairs delay related spatial working memory for a similar task²¹⁵.

Various types of classical conditioning and reinforcement learning paradigms have been used to investigate the role of L-glutamate receptors in non-spatial learning. NMDA receptors have been implicated in visual discrimination and avoidance learning. The competitive NMDA antagonist γ -LGLA impairs learning of an active avoidance task in mice, and blocks post-training improvement of performance of rats, in a Y maze avoidance learning task. The latter has also been demonstrated using CPP^{131,218}. Another NMDA antagonist, NPC 12626, which crosses the blood/brain barrier, impairs learning and recall of an inhibitory avoidance task and spontaneous alternation behaviour in mice^{195,221}. Infusion

of D-AP5 into the hippocampus, amygdala or entorhinal cortex of rats results in amnesia for step down inhibitory avoidance training⁹⁷. MK-801 impairs the performance of rats in aversively motivated complex maze tasks, where foot shock is the aversive stimulus¹⁹⁸. Injection of MK-801 also results in decreased efficiency in obtaining a reward during discriminative reinforcement learning²²⁶. Partial agonists of the glycine site, D-cycloserine and the antibody B6B21, have been shown to significantly improve the rate of acquisition of hippocampus-dependent classical conditioning in rabbits. 7-ClK completely antagonizes this effect²¹⁰. D-cycloserine also reduces learning and memory deficits induced by cholinergic antagonists in rats^{67,193}.

AMPA and metabotropic receptors have also been directly implicated in mammalian learning and memory. Autoradiographic studies have shown that the low affinity component of [³H]AMPA binding increases in several subfields of rabbit hippocampus 3-4 h after training on the "nictitating membrane" classical conditioning paradigm^{211,212}. The increases were attributed to an increased affinity of the low affinity AMPA binding site. [³H]AMPA binding has, also, been found to increase in the rat hippocampus, in response to acute stress²¹⁴. In addition, the AMPA/KA receptor antagonist CNQX results in amnesia for step-down inhibitory avoidance when infused into the amygdala or hippocampus of rats. Memory expression can be blocked by CNQX up to, at least, twenty days after acquisition^{19,97}.

The involvement of metabotropic receptors in learning and memory has been indicated by findings which show that spatial learning potentiates L-glutamate stimulated phosphoinositide hydrolysis in rat hippocampus⁴⁵.

The findings discussed above indicate that NMDA, AMPA and perhaps metabotropic L-glutamate receptors, are important in the processes that underlie learning and memory. Whereas NMDA receptors are involved mainly in memory acquisition, AMPA receptors seem to be more involved in the longer term processes which underlie memory retention.

3.3

Learning and memory related synaptic plasticity

Learning and memory related synaptic plasticity is associated with a cascade of biochemical processes which eventually lead to significant alterations in synaptic morphology. The processes involved in this cascade have been investigated extensively, using both LTP and various learning paradigms as a models. The importance of L-glutamate receptors and increased pre and post-synaptic Ca^{2+} concentrations in the induction of these processes, has been discussed in previous parts of section 3. The key events in the induction of synaptic plasticity and a temporal sequence of the more significant processes in the resulting biochemical cascade are summarized below (see also Figure 2).

Increased high frequency bursting activity of pre-synaptic neurons leads to an initial increase in post synaptic Ca^{2+} concentration, via activation of AMPA and NMDA receptors by L-glutamate^{121,144}. Activation of receptors linked to phosphoinositide hydrolysis, principally mGluRs but also possibly muscarinic acetylcholine(Ach) and μ -opioid receptors, results in intracellular Ca^{2+} mobilization, and perhaps direct PKC activation by diacylglycerol (DAG)^{5,17}. Increases in Ca^{2+} concentration via these mechanisms may also lead to further post-synaptic Ca^{2+} induced Ca^{2+} release¹³³ or influx via voltage gated channels²¹. In addition, activation of adenylate cyclase linked receptors (eg noradrenergic receptors), which results in increased cAMP synthesis and activation of cAMP dependent PKA, may have an important role in the modulation of some processes which influence Ca^{2+} levels^{5,17}.

Increased post-synaptic Ca^{2+} concentration results in the translocation and activation of a number of protein kinases (including PKC) which are responsible for the phosphorylation of various other proteins^{8,17,166,175,188,220}, as well as induction of Immediate-early genes, c-fos and c-jun, whose products form a complex which induces a cascade of 20 to 30 further proteins, many of which are important in synaptic plasticity^{1,3,8,21,51,121,166}, and of a pathway which leads to nitric oxide (NO) synthesis^{25,31,88,152}. These early cascade events lead, in turn to activation of subsequent pre and post-synaptic processes.

Protein kinase activation could also lead to receptor augmentation, by freeing occluded receptors and increasing the number of receptors to which neurotransmitter can bind^{21,114,121,166,188}. As previously mentioned (section 3.1.1), PKC phosphorylates NMDA receptors and PKA phosphorylates AMPA receptors, potentiating their activity^{5,100,132}. Protein kinases also mediate the activation of PLC, PLA₂, and, possibly, other phospholipases¹⁶⁶. These can effect changes in the post-synaptic microenvironment of L-glutamate and other receptors, resulting in alterations in their affinity for neurotransmitters^{16,121,122}. In addition, PLA₂ affects an arachidonic acid (Aa) cascade. Aa, or one of its metabolites, is thought to act as a retrograde messenger^{22,121,161}.

In addition to Aa, the other major major messenger involved in retrograde transmission is nitric oxide (NO), although some recent evidence has indicated the possible involvement of CO^{21,22,31,121,161,233}. Recently NO has received most attention³¹. NO is produced post-synaptically by the action of nitric oxide synthase (NOS) which is activated by elevated levels of Ca²⁺. NO is relatively short lived, but readily diffuses through membranes³¹. It acts pre-synaptically by activating guanylate cyclase, stimulating cGMP formation¹⁵². NO enhances spontaneous transmitter release from cultured hippocampal neurons³¹, and activation of various post-synaptic L-glutamate receptors has been shown to result in pre-synaptic cGMP formation via NO signaling^{63,152}. NO may also directly inhibit NMDA receptors, providing a negative feedback mechanism to prevent excessive or prolonged activation which may lead to neurotoxic effects⁶⁸. It is probable that NO and Aa both act as retrograde messengers, but that NO has an earlier and more transient effect⁹⁰.

Early onset pre-synaptic processes involved in memory formation include increased Ca²⁺ influx through VGCCs⁴⁰, migration of synaptic vesicles towards the pre-synaptic active zone¹⁸² and enhanced transmitter release^{21,53}. Later pre-synaptic processes include phosphorylation of synaptic growth protein (B50/F1/gap43)^{3,21,177} and increases in synaptic vesicle numbers²⁰², as well as synthesis of membrane glycoproteins such as neural cell adhesion molecules (NCAMs), phosphorylation of microtubule associated proteins (MAPs)

and synthesis of structural proteins (eg, tubulin)^{14,21,175} which occur both pre and post-synaptically.

All of these processes presumably contribute to the long lasting potentiation of synaptic efficacy which is associated with the later stages of learning and memory related synaptic plasticity^{73,74,118,119,120}, by strengthening and enlarging existing synaptic connections as well as leading to the formation of new synapses^{21,121,166,177,202}. Many of the memory associated biochemical, pharmacological and morphological processes mentioned in this section, have been established and arranged in temporal sequence using the paradigm of one-trial passive avoidance training in the domestic chick. This, as well as a number of other paradigms will be discussed in section 4.

4

The domestic chick, *Gallus domesticus*, as a model in learning and memory related synaptic plasticity.

The evidence for the involvement of L-glutamate receptors in learning and memory discussed so far, arises mainly from investigations using adult mammals. The chick, however, is a precocial animal, in which any synaptic changes, associated with memory and learning, will be superimposed upon changes which occur due to the rapid development of the chick CNS. Use of suitable controls, however, allows any observed changes to be reasonably attributed to memory and learning related plasticity, although these may be quite small compared to overall changes associated with development. Also, there are a number of advantages, in using the chick to investigate such memory and learning related changes, rather than adult animals.

These include¹⁷⁴:-

1. The chick has the well organized and predictable behavioural repertoire of a precocial animal.
2. It has a large and well developed brain, and a relatively high brain/body ratio.

3. It has a reduced blood-brain barrier, enabling rapid diffusion of injected substances into the brain.
4. Its thin, unossified, skull makes intracerebral injections relatively easy and also makes removal of the brain, for *in vitro* work, a relatively quick and simple task.

The major drawback in using chicks for this type of research, is that their neuronal circuitry is relatively poorly understood in comparison with that of the mammalian CNS.

4.1

Avian learning paradigms

The principle avian learning paradigms that have been employed in recent times, to study various aspects of learning and memory in a number of species, include imprinting⁹⁴, passive avoidance training^{38,175,202}, pebble floor discrimination⁴, food storage¹⁰⁵ and song learning¹⁴⁹. In studies involving the use of the domestic chick, the former three paradigms have been most important, although a number of other specific learning tasks have been used⁴.

4.1.1: *Imprinting.*

Precocial birds, such as young chicks, learn very quickly to recognise the visual and auditory characteristics of their mother. This information is rapidly encoded and very strong approach behaviour is quickly initiated. When this “social attachment” occurs the young bird is said to be imprinted upon its mother. The imprinting stimulus for a precocial bird does not necessarily need to be its mother. Young chicks will approach a wide range of objects during a “sensitive” period which begins a number of hours after hatching. Continued exposure to a particular object results in imprinting, and chicks show signs of contentment when they are in close proximity to it. If the familiar object is removed, however, the birds become distressed and may even panic. Examples of objects which have been used as imprinting

stimuli in experiments include internally illuminated, rotating coloured boxes, or cylinders, and stuffed jungle fowl¹⁹⁴.

The imprinting paradigm involves a number of training trials, up to one hour in length, where a dark reared chick is placed on a running wheel and exposed to an imprinting object. Control chicks are handled as much as trained chicks but are kept in darkness. After a further period (up to 8 hours) in darkness, both trained and control chicks are tested by simultaneously exposing them to two stimuli, one of which is the imprinting stimulus. The approach behaviour of the chicks is then observed and the level of recall for the imprinting stimulus is recorded. A number of biochemical and other correlates of imprinting memory, including changes in binding to L-glutamate receptors, have been found using this paradigm^{98,126}.

4.1.2: *Passive avoidance training.*

The one trial passive avoidance training paradigm was first introduced by Cherkin in 1969, and has since been developed by Rose and co-workers^{38,175,202}. Young chicks instinctively peck at a range of small objects and quickly learn to discriminate between what is edible and inedible. If they peck at an unpalatable object, such as their own faeces, they quickly learn to avoid similar objects. Training initially involves a pre-training stage, where chicks are offered a small brightly coloured bead (usually white) at which they peck spontaneously. Only those birds which exhibit a natural tendency to peck are trained further. When a chick is presented with a bead which has been dipped in an unpleasant tasting substance, methylantranylate (MeA), it pecks and exhibits a disgust response. It will, subsequently remember not to peck the bead for many days after training. Control birds are presented with an identical bead, dipped in water (W), and these, subsequently, continue to peck the bead when it is presented. This passive avoidance task combines aspects of both of the associative learning categories mentioned in section 3. It can be described as classical conditioning in that chicks learn to associate a visual stimulus with an unpleasant taste, and instrumental conditioning in that the innate pecking response of chicks is inhibited¹⁸⁴.

Various biochemical, pharmacological, physiological and morphological aspects of neuronal plasticity (discussed in 4.3) can be measured in MeA and control chicks, and any significant differences between the two groups can be reasonably attributed to a memory and learning related response^{175,177,202}. Experiments involving birds trained using MeA and then rendered amnesic, using trans-cranial subconvulsive electro-shock, can also be performed in order to show that such differences are learning and memory associated, and do not result from stress due to the bitter taste, or from any other confounding variables, such as the possible appetitive nature of water^{157,180}.

One of the main advantages of passive avoidance training, over other chick training paradigms such as imprinting, is the precision of timing that is possible. The one trial event involved can be precisely observed and timed and, therefore, an insight can be gained into the time course over which learning and memory related processes occur. Also, for *in vitro* work at least, birds can be killed a relatively short time after training¹⁷⁴. These features of passive avoidance training have allowed a great deal to be learned about memory and learning related plasticity in the chick brain (see 4.2). Serrano et al¹⁹⁰ have used pharmacological interventions to develop a model of memory for passive avoidance training which involves three serially dependent stages, short, medium, and long term memory. Data accumulated by Rose and co-workers over a number of years, however, provide strong evidence for a model involving two waves of processes, suggested to be involved in memory formation and consolidation¹⁷⁷ (see 4.2).

4.1.3: *Pebble floor discrimination*

Another learning task which involves pecking at small objects, and takes advantage of the ability of young chicks to discriminate between palatable food objects and unpalatable objects, is the pebble floor discrimination task⁴. This task involves placing a chick on a floor where small pebbles have been firmly affixed and food grains of a similar size and appearance have been scattered amongst the pebbles. The chicks quickly learn to distinguish

between the food and the pebbles, and the rate at which the number of pecks at pebbles declines is taken as a measure of learning⁴.

4.2

Regions of the chick forebrain involved in learning and memory.

The chick telencephalon is largely composed of "sheets and clusters of cells"⁹⁴ each of which can be considered to be a discrete forebrain region (see Figure 3). A great deal of evidence indicates that memory and learning related plasticity in the chick forebrain is localized in a few specific regions, and occurs mainly in the left hemisphere. Many of the associated physiological, biochemical and morphological processes (section 4.2.2) have been demonstrated in these regions^{175,177,202}. Most investigators have concentrated on two regions, the intermediate and medial hyperstriatum ventrale (IMHV) and the Lobus parolfactorius (LPO), which were initially implicated in learning processes by investigations of 2-deoxyglucose (2-DG) accumulation using passive avoidance¹⁷⁸, and uracil incorporation using imprinting⁹⁴. The importance of these regions has since been supported by a great deal of evidence from investigations of many other correlates of learning and memory^{75,175,177,190} (section 4.3). Other regions, however, such as the paleostriatum augmentatum (PA)²⁰³, the lateral neostriatum (LN)¹⁵⁹, the hippocampus^{184,228} and the archistriatum¹¹² have also been implicated.

The IMHV has been described as an avian equivalent of part of the mammalian neocortex^{42,94,167} and seems to be important in visual integrative processing. In each hemisphere it receives visual signals indirectly from the tectofugal and thalamofugal pathways, as well as from direct bilateral connections with the optic tectum. It is connected reciprocally to the intermediate and dorsal part of the archistriatum (AId), the hyperstriatum accessorium (HA) and the PA, and receives bilateral connections from the hippocampus (Hp) and dorso-medial septum (Se). It also receives ipsilateral connections from the medial neostriatum (MN), the hyperstriatum dorsale (HD), the hyperstriatum intercalatum

supremum (HIS), area perihippocampalis (APH) and thalamic nuclei. It projects to the LN and posterior archistriatum⁹⁴.

The LPO, PA, paleostriatum primitivum (PP) and nucleus accumbens together comprise the avian basal ganglia or paleostriatal complex. These regions are considered to be equivalent to the mammalian striatum, and are believed to be similarly involved in motor responses^{167,202}. They are also thought to be involved in motivational behaviour¹⁴⁸. The LPO, which is assumed to be analagous to the caudate nucleus, is closely associated with the olfactory bulb and may also have an important role in processing the sensory information involved in smell and taste^{94,167}.

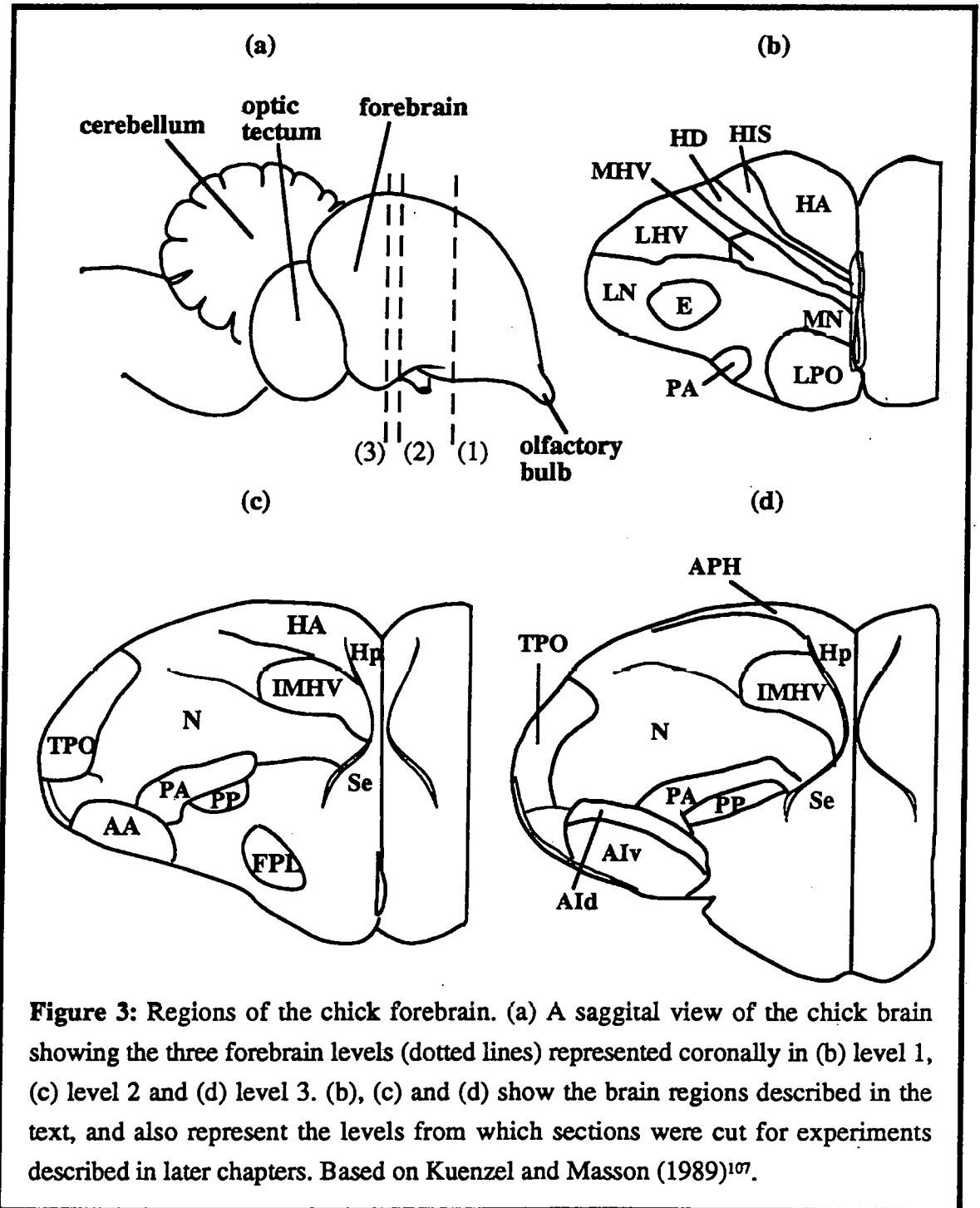
Some light has been thrown on the roles of various brain regions, the IMHV and LPO in particular, in chick memory and learning by studies involving the lesioning of specific regions before or after passive avoidance training^{75,175}. These have shown that the left IMHV is necessary for memory acquisition, whilst the LPO (bilaterally) is involved in memory consolidation. In the absence of the LPO, however, the right IMHV can assume a similar role. This suggests a "flow" of information, between the IMHV and LPO during long term memory formation. There are, however, no known, direct neural connections between the IMHV and LPO⁷⁵. If such a "flow" model is valid, therefore, intervening regions (eg, the archistriatum) must pass information between them. Other models of memory, eg, where information is processed simultaneously by different regions, would not necessarily require such connections.

4.3

Correlates of passive avoidance training in the chick.

In section 3.3, the known physiological, pharmacological, biochemical and morphological correlates of learning and memory related synaptic plasticity were summarized. The processes which occur during a similar cascade of intra and inter-cellular events in the chick brain after passive avoidance training, have been studied extensively by

Rose and co-workers, and (as mentioned in section 4.1.2) apparently occur in two “waves” after training¹⁷⁷. Most of these processes have been shown to occur in either the IMHV, the LPO or both, although other regions have been investigated more recently.



Physiological correlates of passive avoidance training include increases in high frequency bursting activity in neurons of both the IMHV and LPO. These have been shown

to be time dependent and memory specific^{73,74,118,119,120}. In the IMHV significant increases in bursting were recorded 3-4, 5-6 and 6-7 h post-training, but not at earlier or later times. Bursting in the right IMHV was significantly higher than in the left at 6-7 h post-training⁷³. In the LPO significant increases in bursting were recorded 4-7 h post-training, but no significant increases were found at 1-4 or 7-10 h post training⁷⁴. These data were shown to be memory specific using electro-shock experiments such as those described in section 4.1.2, and suggest a role for both the IMHV and the LPO in the consolidation phase of memory for the passive avoidance task^{73,74}.

At the level of pharmacology, the involvement of a number of neurotransmitters and their receptors in memory for passive avoidance training has been demonstrated. This includes L-glutamate and its receptors (NMDA receptors in particular)³⁵ but these will be discussed in detail in section 4.4. Other receptor types whose activity has been correlated with passive avoidance training include δ -opioid receptors. Binding of the selective δ -opioid ligand [³H](D-Pen²,D-Pen⁵)-enkephalin (DPDPE) was found to increase in the right PA and bilaterally in the LPO, 30 min after passive avoidance training⁵⁰. Transient alterations in the level of binding to muscarinic cholinergic¹⁷⁹, GABAergic²⁹ nicotinic cholinergic and serotonergic² receptors in the chick forebrain have also been demonstrated.

An important biochemical signaling process, involved in the early stages of memory formation for the passive avoidance task, involves increased Ca²⁺ entry into neurons via VGCCs. Injection of the specific N-type VGCC antagonist ω -conotoxin GVIA (Cg-TX) into the left or right IMHV prior to passive avoidance training, results in amnesia for the task when chicks are tested 30 min or 3 h post-training. This suggests that entry of Ca²⁺ into neurons via N-type VGCCs is necessary for memory acquisition and that the IMHV is involved bilaterally. Increased uptake of Ca²⁺ into prisms of chick forebrain tissue, taken from either the right or left IMHV, has been demonstrated to occur at 30 min after passive avoidance training, but not at 5 min or 24 h. This training induced increase can be inhibited by Cg-TX in the left but not the right IMHV. This suggests that some other VGCC may be involved in addition to N-type channels, in the right IMHV. These could possibly be P or Q-

type as injection of L-type VGCC inhibitors into the IMHV does not result in amnesia⁴⁰. Pre-synaptic Ca^{2+} entry is intimately associated with neurotransmitter release¹⁷², so it is possible that that Ca^{2+} entry into neurons of the IMHV could also be associated with transmitter release. Indeed, a significant increase in K^{+} induced, Ca^{2+} dependent glutamate release in the left IMHV 30 min after passive avoidance training has been demonstrated. A similar passive avoidance training increase in glycine release, at the same time point, was found to be Ca^{2+} independent⁵³. In addition, it has been shown that the spatial arrangement of pre-synaptic vesicles in neurons of the IMHV becomes rearranged, with a greater number of vesicles closer to the pre-synaptic active zone, 30 min after passive avoidance training¹⁸².

Retrograde messengers such as nitric oxide and arachidonic acid have also been shown to have a role in learning and memory for the passive avoidance task. Bilateral, pre-training injections of the NO synthase inhibitor, L-nitro arginine, into the IMHV results in amnesia for passive avoidance training when the chicks are tested 30 min or 1 h afterwards^{88,89}. Similar pre-training injections of nordihydroguaiaretic acid (NDGA) or aristolochic acid (AST) which inhibit PLA₂, a key enzyme in Aa release, also result in amnesia for passive avoidance training. The earliest time at which amnesia is apparent following these injections, however, is 1.25 h post-training, somewhat later than the onset of amnesia after NOS inhibition⁹⁰. If NO and Aa both act as retrograde messengers, it seems likely that fast diffusing NO acts as an early transient messenger, whereas Aa, or at least one of its metabolites (the effects of which have a slower time course) acts later but with a more long lasting effect.

Another factor which is known to be important in the learning and memory associated biochemical cascade is PKC translocation and activation^{8,12,220}. The PKC inhibitors, H-7 and melittin cause amnesia for passive avoidance training when injected immediately after training, at concentrations which prevent the phosphorylation of substrate proteins *in vitro*, and when chicks are tested 30 min post-training. This effect is only apparent when injections are directed into the left, but not the right, IMHV. (The situation with NMDA receptor antagonists is similar-see section 4.4). Also, passive avoidance

training results in a specific decrease in phosphorylation of the pre-synaptic growth protein, B50/F1/gap43, which is a substrate of PKC¹⁷⁷).

Synaptic remodelling processes which lead to the various morphological changes associated, mainly, with the later stages of memory related synaptic plasticity, are known to depend upon synthesis of new proteins^{175,177}. An important stage in this protein synthesis is the induction of the immediate-early genes (IEGs), *c-fos* and *c-jun*, which in turn results in further gene expression. The induction of these IEGs has been correlated with the passive avoidance task and is observable 30 min post-training^{6,175,177}. Antagonism of corticosteroid receptors, which are known to modulate gene expression, also results in amnesia for the task¹⁸³. Among the proteins whose synthesis have been shown to increase as a result of passive avoidance training are the structural protein, tubulin and various glycoproteins, including the neural cell adhesion molecule, NCAM¹⁷⁷. Injection of the glycoprotein anti-metabolite, 2-deoxygalactose¹⁴, 30 min post-training or 6-8 h post-training resulted in amnesia for the passive avoidance task. Similar injections administered 2-6 h or after 8 h post-training had no effect. Similar injections of the antibody, anti-NCAM were amnesic only when administered during the 6-8 h time interval. This shows that two waves of glycoprotein synthesis, and therefore, two waves of synaptic plasticity, are required for the establishment of long term memory for passive avoidance training in the chick, and that NCAM is only involved in the later of these. It is possible that this late wave of synaptic plasticity involves the modulation of cell-cell adhesion processes which reflect the selection and stabilization of synapses which are responsible for the maintenance of long term memory¹⁸⁷.

Many changes in synaptic morphology have been shown to occur in various regions of the chick brain, particularly the IMHV and LPO, 24 h or more after passive avoidance training. These include: increased numbers of synapses, increased dendritic spine density, increased spine head diameter, increased post-synaptic thickening length and increased pre-synaptic bouton volume²⁰². Moreover, many of these changes have been shown to be memory-specific using electro-shock experiments¹⁵⁷. More recently, changes in synaptic

morphology have also been shown to occur at a much earlier stage after passive avoidance training. A large increase in synaptic number was shown to occur in the right IMHV only 1 h post-training, although the average post-synaptic density size was 57% smaller⁵⁹. This suggests that new synapses may have been created by splitting of existing synapses. Even earlier, at 30 min post-training, pre-synaptic active zone lengths have been shown to increase in addition to the rearrangement of synaptic vesicles mentioned earlier in this section¹⁸².

Many of the biochemical correlates of learning and memory related synaptic plasticity are, generally, seen as "housekeeping" processes, which are necessarily associated with, but do not directly subserve, memory and learning¹⁷⁴. Memory is seen as, somehow, resulting from a remodelling of neuronal circuitry within the brain. Changes involving the pharmacology of L-glutamate, and perhaps other, receptors, in various brain regions could, however, directly subserve memory by modifying post-synaptic sensitivity to L-glutamate, and other neurotransmitters. Increased receptor density within a particular chick brain region could, in fact, indicate where new synapses are being formed¹⁷⁴. So, if changes in the number or affinity of receptors can be found to occur at various time points after a learning task such as passive avoidance training, in well defined brain regions, it could provide an important insight into the regionality and time course of memory acquisition and retention.

4.4

L-glutamate receptors in the chick brain.

The distribution of various L-glutamate receptor subtypes, in the chick brain, has been mapped, using quantitative autoradiography, by Henley et al (1989)⁸⁴. Under the conditions used in this study, the regional distributions of [³H]L-glutamate, [³H]AMPA and [³H]KA binding, were shown to be distinctly different.

[³H]L-glutamate binding was shown to be densely localised in the telencephalon, particularly in the neostriatum. Other areas labelled, included the thalamus, nucleus mesencephalicus lateralis pars dorsalis, the superficial layers of the optic tectum and the

molecular layer of the cerebellum. [^3H]AMPA binding sites were distributed, relatively homogeneously throughout the telencephalon, but were most densely localised in the hippocampus. The striatum griseum et fibrosum superficiale of the optic tectum, and the molecular layer of the cerebellum were also labelled. [^3H]KA binding sites were highly regionalized within the telencephalon. It bound, relatively densely to the hyperstriatum ventrale(HV) and the hyperstriatum dorsale(HD), and less densely to the hyperstriatum accessorium(HA). It was negligible in the neostriatum. The densest binding to any region in the study, was of [^3H]KA to the molecular layer of the cerebellum. The thalamus was also labelled.

NMDA was found to inhibit a large proportion of [^3H]L-glutamate binding to all telencephalic areas, whereas [^3H]L-glutamate binding was found to be only marginally sensitive to AMPA and KA. This would indicate that, under the conditions used, [^3H]L-glutamate binds, mostly, to NMDA sensitive sites. [^3H]MK-801 binding was found to closely resemble the distribution of NMDA sensitive binding, with the notable exceptions of the ectostriatum and paleostriatum, which are labelled more densely by NMDA sensitive [^3H]L-glutamate than by [^3H]MK-801, and the cerebellum where little or no [^3H]MK-801 binding is apparent. This would suggest that an NMDA receptor isoform which has very low affinity for MK-801, similar to the mammalian cerebellar isoform (section 2.1.2), could be present in the chick forebrain in addition to the cerebellum. The distribution of competitive antagonists were not determined in the study by Henley et al(1989)⁸⁴.

In a further autoradiographic mapping study, Henley and Barnard mapped the distribution of, the non-NMDA antagonist, [^3H]CNQX in the chick brain. The binding distribution of [^3H]CNQX was found to be very similar to the combined distributions of [^3H]AMPA and [^3H]KA binding⁸².

Similar receptor distributions were observed by another group (Mitsacos, et al, 1990¹³²) where the displacement of [^3H]L-glutamate binding, by NMDA and QA was measured. Also, these experiments indicated that no significant differences in binding

occurs, in the presence, or absence, of Cl⁻ ions, in any telencephalic region, except the ectostriatum. More recent work²³¹ has confirmed the distribution of [³H]CNQX binding found by Henley and Barnard, and has also demonstrated that both AMPA and KA displace [³H]CNQX in a biphasic manner, indicating the presence of more than one binding site for both ligands²³¹.

Compared to the situation in mammals, little progress has been made in determining the structure of avian L-glutamate receptors. An NMDA receptor subunit (NR1) has, however, been cloned from duck brain. The N-terminus 898 amino acid residues of this protein have a very high degree of homology with the rat NR1a and d isoforms. In the 66 amino acid residues at the C-terminus, however, this homology is completely lost, although detailed analysis of the amino acid sequence of avian NR1 revealed that the receptor architecture is more or less the same as in mammals. *In situ* hybridization revealed that NR1 is distributed throughout the duck brain but is expressed most prominently in the HV, LPO and hippocampus¹⁰⁸.

Evidence for the involvement of L-glutamate receptors in chick memory and learning, has largely arisen, not surprisingly, from studies using either passive avoidance training or imprinting. Homogenate binding assays, performed on IMHV tissue from chicks trained on an imprinting task, showed that a significant increase in NMDA sensitive [³H]L-glutamate binding occurred 8-5 hours after training, in the left IMHV. The concentrations of [³H]L-glutamate used were virtually saturating, so the increase could be attributed to an increase in the total number of NMDA receptors rather than an increase in their affinity^{95,126}. In a similar, but more recent study, in which the equilibrium binding kinetics of [³H]L-glutamate was examined in chicks after imprinting, both the maximal binding and the affinity of receptors for [³H]L-glutamate was shown to increase 7-8 h after imprinting⁹⁸. This increase in affinity could result from [³H]L-glutamate binding to non-NMDA receptors. Increased numbers of NMDA receptors at this time point would suggest a role for NMDA receptors in memory retention.

It has been demonstrated, using passive avoidance training, that NMDA receptors are involved in memory acquisition and the early stages of memory formation, as would be expected from their role in the induction of LTP like activity (section 3.1.1). Pre-training or 5 min post-training intracerebral injection into the left or right IMHV, or intraperitoneal injection, of MK-801 resulted in amnesia for passive avoidance training in chicks tested 3 or 24 h afterwards, whilst similar injections of the non-NMDA antagonists, CNQX, DNQX and NBQX had no effect on memory for the task³⁵. In a more recently reported experiment, however, DNQX has been shown to result in amnesia for a similar passive avoidance task 80 to 90 min after training, when administered 10 or 25 min after training¹⁶⁹. Pre-training injection of 7-ClK, a highly selective antagonist of the glycine site on the NMDA receptor, into the left, IMHV also resulted in amnesia for the passive avoidance task when chicks were tested 1 or 3 h post-training. Right IMHV injections had no effect²⁰⁰ (see chapter 4 for details). Quantitative autoradiographic studies have shown that increases in NMDA sensitive [³H]L-glutamate, but not [³H]AMPA, binding occur 30 min after training, and that these increases are localized to the left IMHV and left LPO²⁰⁵ (Chapter 3).

Metabotropic L-glutamate receptors have also, recently, been implicated in memory for the passive avoidance task. Injection of both L-AP4, and the more specific mGluR antagonist MCPG, into the IMHV result in amnesia for the passive avoidance task. L-AP4 was amnesic when injected both pre-training and up to 2 h post-training. After pre-training injections, amnesia first became apparent 1 h post-training. Only pre-training MCPG injections were effective in causing amnesia, the onset of which occurred 3 h post-training⁸⁷. These results are in accordance with the postulated role of mGluRs in associative LTP (section 3.1.1), where mGluR antagonists inhibit maintenance of the later stages²¹.

The findings summarized above strongly suggest that L-glutamate receptors, and particularly NMDA receptors, play a crucial role in the formation of memory for passive avoidance training, and it is possible that AMPA receptors play a role in consolidation and maintenance. It is clear, however, that if chick L-glutamate receptors are similar to those in the mammalian brain, the situation is likely to be much more complex. It is interesting to

speculate whether NMDA receptor subtypes could have differing roles in memory formation after passive avoidance training, if indeed these subtypes can be distinguished in the chick forebrain. There is certainly some evidence that such a differential role is possible in mammals.

Changes in the lipid microenvironment of AMPA and NMDA receptors, resulting from PLC treatment, have been shown to induce changes in the affinities of the two subtypes for their respective ligands^{121,122}. PLC treatment was shown to have no effect on the affinity of NMDA receptor sites for [³H]L-glutamate and glycine, while the affinity of these receptors for [³H]CPP and [³H]TCP (channel blocker) was found to be decreased. The affinity of [³H]AMPA binding can be modulated by similar treatment using PLC or PLA₂^{16,121,122}. These findings indicate a significant difference in the roles of nNMDA and cNMDA receptor subtypes in mammalian synaptic plasticity, and also indicate that changes in the affinity of the channel blocker site may be important. They also support the body of evidence for the importance of AMPA receptors in mammalian synaptic plasticity.

For the most part, the work done so far on the involvement of L-glutamate receptor subtypes in learning and memory following passive avoidance training has been concerned with the early stages of memory formation, and has concentrated on only two regions, the IMHV and the LPO. This leaves a great deal of room for investigation of the role, if any, of these receptors at later time points after passive avoidance training and, possibly, in other regions.

The research project described in the remaining chapters of this thesis aims to further establish the role of L-glutamate receptors in the formation and/or retention of memory for passive avoidance training in the chick. It will clarify their mode of regulation by examining whether changes in their affinity or their total number (or, more strictly, concentration) are affected by passive avoidance training. It also proposes to examine the distinction, if any, between the roles of L-glutamate, and particularly NMDA, receptor subtypes. Also, the regional distribution of alterations in binding to L-glutamate receptors at selected time points after training, should help to clarify which brain regions are involved in different stages of

learning and memory. These aims were investigated using the techniques of quantitative receptor autoradiography and behavioural pharmacology described in chapter 2.

Chapter 2

Investigating L-glutamate receptors in chick memory formation: techniques and data analysis

5

Experimental techniques

The role of L-glutamate receptors in learning and memory has been established using a variety of behavioural pharmacological techniques, chosen to suit the species and learning paradigm involved (chapter 1, 3.2 and 4.4). These techniques are essentially designed to find how interventions using drugs affect learning and memory for a particular paradigm. The simplest and most direct intervention involves direct injection of a drug into the brain. In the chick this is a relatively simple, quick and painless procedure which has been used to elucidate the roles of many correlates of passive avoidance training (chapter 1, 4.3-4) including those involving L-glutamate receptors^{35,87}. In experiments described in chapter 4, this technique was used to confirm the role of NMDA receptors in the early stages of learning for the passive avoidance task and to investigate the importance of the glycine co-agonist site. In those described in chapter 6 it was used to investigate the role of AMPA receptors in later stages of memory for this task. In section 5.2 the general procedures involved in administering intracerebral injections will be described.

Quantitative receptor autoradiography, *in vitro*, can be used very effectively to measure the binding of various radioligands to their receptors²⁰⁴, and is ideal for the investigation of changes in the equilibrium binding parameters of L-glutamate after passive avoidance training in the chick²³¹. It has also been used to show the regional distribution of various receptor types in brain^{84,138}, and has allowed the identification of receptor subtypes¹³⁶. In section 5.3 the general procedures for chick brain tissue preparation,

radioligand binding and quantitative autoradiography are summarized. These are the major techniques involved in the experiments described in chapters 3, 5 and 6.

Section 5.1 will describe the rearing and housing conditions of the chicks used in all of the experiments reported in this thesis.

5.1

Rearing, housing and training conditions of chicks.

For all of the experiments described in chapters 3 to 6, fertile eggs of a commercial strain (Ross Chunky) were incubated in a communal incubator at 38-40°C under a 12 h light/12 h dark cycle (lights on at 07.00 h) for 21 days until they hatched (day 0). Thereafter, chicks were held in a communal brooder under identical conditions for 30 ± 6 h. All experiments commenced at 08.30 h when pairs of chicks were placed in 20 x 25 x 20 cm aluminium pens (floored with clean blue paper towel), each of which was illuminated with a 25 W red light. One chick in each pair was marked by a spot of blue animal dye and the chicks were allowed to acclimatize to these conditions for 1 h prior to pre-training. Where chicks were to be left in the pens for more than six hours, chick crumb was scattered on the floor of each pen prior to pre-training and where chicks were to be left in the pens overnight, a small tub of water was also placed in each. Pre-training involved three, 10 s presentations of a 2.5 mm diameter white plastic bead. Training and testing were performed a by single, 30s, presentation of a 4 mm diameter bright chrome bead, MeA coated for training, water coated for autoradiography controls, dry for testing.

5.2

Behavioural pharmacology

In this thesis all of the behavioural pharmacological experiments described involve intracerebral (ic) injection of drugs directly into the IMHV. These were performed using a Hamilton Syringe fitted with a plastic sleeve as a stop, which allows an injection depth of 4

mm. Injections were directed using a plexiglass headholder⁵⁷ containing bore holes, which guide the injection into the defined stereotaxic coordinates of the left or right IMHV^{35,87}. In all cases the injection volume was 5 μ l per hemisphere. All drugs injected were dissolved in 0.9% sterile saline, prepared by dissolving 0.9 g of NaCl in 100 ml of distilled water. This was then divided into 3 ml aliquots and autoclaved to sterilize. All chicks in these experiments were trained on an aversive stimulus (MeA), control chicks were injected with 0.9% sterile saline alone.

5.3

Quantitative receptor autoradiography

The general procedures used in all of the experiments involving radio-ligand binding and quantitative autoradiography in this thesis were as follows^{204,205}

5.3.1: Training and tissue preparation

Chicks were trained and tested, using the passive avoidance training paradigm described in chapter 1 (section 4.1.2), under the conditions described in section 5.1. MeA chicks, which refused to peck, and W chicks, which continued to peck (see section 4.1.2), on testing with a dry chrome bead were killed by decapitation (normally ~ 85 % for both). The brains were dissected out and divided into forebrain and hindbrain portions using guide lines in a brain mould¹⁷⁸. The brain portions were then rapidly frozen in an isopentane/dry ice(CO₂(s)) mixture, wrapped in foil (coded to ensure that the experiments were performed blind) and, temporarily stored in dry ice. These were then placed in polythene bags and stored at -70°C in a freezer, until required. Brains can be stored at this temperature for several weeks before deterioration of tissues becomes problematic²⁰⁴.

5.3.2: Sectioning

The required brain portion, in this case forebrain, was mounted on a cryostat chuck, and 10 μ m coronal sections were cut in a Reichert cryostat at approximately -20°C (the ideal

cutting temperature can vary, depending on external conditions). Sections were cut at an angle equivalent to that at which the brain was divided in the brain mould. This is close to the stereotaxic angle, and allows easy identification of brain regions (see chapter 1, figure 3). Sections were thaw-mounted onto poly-L-lysine subbed coverslips (subbed by immersion in 10% poly-L-lysine solution for at least 10 min, then fan drying for a minimum of 3 h) and dried in a cold air stream for 5 min. If not used immediately, the sections were placed in folders and polythene bags, and stored at -20°C in a freezer until required.

5.3.3: Incubations

After being brought to room temperature sections were pre-incubated for 1 h, in 50 mmol l⁻¹ tris-citrate buffer (pH 7.4) (TCB), to remove endogenous ligands, and then dried in a cold air stream. TCB was prepared by dissolving 60.57 mg of tris(hydroxymethyl)methylamine in 10 litres of distilled water, then adding small amounts of a saturated citric acid solution until pH fell to 7.4, as indicated by a Phillips PW 2421 pH meter. After pre-incubation, the required radioligand solution (or radioligand/displacer for non-specific binding), in TCB was applied as a 100µl drop, to completely cover each section. Sections were then incubated at 4°C or room temperature, depending on the radioligand, for the time period necessary for binding to reach equilibrium, eg, 20 min for [³H]L-glutamate at 4°C²²⁹. Incubation was stopped by washing sections, three times in ice cold TCB. The most effective washing times were determined using incubations as described above, but where washing times are varied. The optimum washing time is that where specific binding is not significantly reduced with respect to non-specific binding (NSB), but where NSB is as low as possible. Optimum washing time varies from radioligand to radioligand, and the times used in the experiments described in this thesis were determined for previous work (eg, previously used washing times have been- 5 s for [³H]L-glutamate, 30 s for [³H]MK-801, 2 s for [³H]AMPA and 15 s for [³H]glycine)²⁰⁵. After washing in TCB sections were dipped in ice cold distilled water, to remove buffer salts, and then dried in a cold air stream.

5.3.4: Liquid Scintillation counting

In experiments not involving autoradiography, each coverslip, with radioligand bound to the mounted section, was broken into a scintillation vial, 8ml of liquid scintillant (Packard emulsifier) was added to each vial, and the number of degradations per minute (dpm) determined using a Beckman LS 7500 scintillation counter. This indicates the amount of radioligand bound to each section. The accurate concentrations of radioligand solutions used were determined similarly, where 25 μ l of solution followed by 8 ml of scintillant was added to each of three vials, for each radioligand. The concentration of the [3 H]radioligands in nmol l⁻¹, can be calculated using the following equation.

$$1. \quad \frac{\text{mean activity of 25 } \mu\text{l of sample (dpm)} / \text{specific activity}}{1000} \times 40$$

A more direct way of performing this calculation is to simply divide the mean activity by 25 times the specific activity (s.a) of the sample.

5.3.5: Preparation of brain paste standards

During densitometric analysis of autoradiograms it is necessary to generate a calibration curve against which optical density can be measured, in order to quantify the level of radioligand binding to a particular section. This can be done using autoradiograms of pre-prepared brain paste standards. These are 3 H-standards with known levels of radioactivity²⁰⁴. The brain paste standards for the experiments described in subsequent chapters were prepared according to the method of Unnerstall et al (1982)²¹⁹.

The grey matter from 15 one day old chick forebrains was homogenized to a paste, in low viscosity silicone oil to prevent frothing, using a polytron (setting 4, 30-40s, 4°). 3 H-leucine was diluted 100 μ l to 200 μ l with distilled water and this was followed by six 50% serial dilutions. 0.5 g of homogenized forebrain tissue were transferred into each of 12 standard 1.5 ml Eppendorf tubes which were then centrifuged in a Beckman microfuge until the tissue settled at the bottom. Each dilution of 3 H-leucine was then split into two of the

Eppendorf tubes to give six duplicate standards. Each standard was thoroughly mixed, by hand, for at least 10 min using disposable plastic stirring rods, to insure that the radioactivity was evenly distributed in each standard, then centrifuged at 3000 rpm for 3 min to remove air bubbles. All 12 tubes were then simultaneously placed in a freezer at -70°C and left over night. Each of the frozen standards was removed from the Eppendorf tube by dipping in water (at room temperature) and removing the base of the tube using a razor blade. The standard could then be pushed out onto a cryostat chuck and mounted with Tissue-Tek. 10µm sections of each standard were thaw mounted onto coverslips, 6 standards on each coverslip. Sample sections were taken at intervals, 6 from each Eppendorf tube or 12 from each duplicate standard, and their activities were measured by liquid scintillation counting. The mounted standards were fan dried for 5 min and stored at room temperature. In one experiment 17 sets of standards were apposed to ³H-Ultrafilm (see section 5.3.6) and when developed their areas were measured using the image analysis system described in section 5.3.7. The average of these areas was then used to calculate the activity of each standard in fmol mg protein⁻¹. This calculation assumes 8% protein content and is as follows for each standard: average dpm value divided by average area, multiplied by thickness of standard (10µm), divided by specific activity (in dpm) of radioligand used for binding to sections and multiplied by 12.5 to account for assumed 8% protein. It is also assumed that the specific activity of chick brain tissue is 1 and, therefore, has no influence on results.

5.3.6: Autoradiography

For the preparation of autoradiograms, coverslips with mounted sections and bound radioligand, were affixed to card using appropriate adhesive (UHU glue), along with an additional coverslip with mounted brain paste standards. Each piece of card used was comparable in size to the sheet of film, to which it was apposed. Cards, with mounted coverslips, were apposed to LKB ³H-Ultrafilm, under dark room conditions, and were secured between sheets of aluminium with sticky tape. They were then sealed in black photographic bags and stored at 4°C. Exposure time depends on the activity of the bound radioligand, estimated by measuring dpm of radioligand bound to additional sections, and

can be up to 60 days. Accurate determination of the time at which autoradiograms should be developed was achieved using test strips, prepared in conjunction with the autoradiograms (again using additional sections). Autoradiograms were developed under dark room conditions, using AGFA G150 developer (4 min 20°C) and Ilford hypam fixer (3 min), and were then washed for at least 1 h in distilled water and left to dry.

5.3.7: Densitometry and image analysis.

Densitometry was performed on autoradiograms using a Joyce-Loebl Magiscan MD image analysis system (colour plate 1, page - xiii). Any areas of ^3H -Ultrafilm which have been exposed to radiation accumulates silver grains on development, increasing the optical density of these areas. The greater the radioligand binding density to a chick forebrain section, the greater the optical density of the apposed region of film. The optical density of brain regions, on an autoradiogram, can be related to radioligand binding density, using a calibration curve of optical density against activity level. Such calibration curves were generated using the "Magiscan" system, by measuring the optical density of brain paste standards, using liquid scintillation counting as described in section 5.2.5. Optical density measurements of brain regions were then made (examples of images from which measurements were made are shown in colour plate 2, pages - xiv and xv). These were automatically related to activity level, via the standard curve, and expressed in the units fmol mg protein⁻¹. Data was stored on a hard disk, and printed out for reference. It was then transferred from the hard disk, to an apple Macintosh system, using the "kermit" software package during earlier experiments (chapter 3) and simply transferred by copying onto floppy disk (made Macintosh compatible by access PC software) during later experiments (chapters 5 and 6). Once saved as a Macintosh file, various operations can be performed on the data, eg, subtracting non-specific binding (NSB) from total radioligand binding to find specific binding values, standardization of data, statistical analysis, etc. Data standardization is usually necessary, in order to correct for variation in total binding, between brains. Some variation in the overall optical density of individual sections on each film results not only from inter animal variation (a major factor) but also from error arising from the incubation

procedure. Small variations in radioligand or displacer concentrations and in washing times can have an appreciable effect on the overall binding to a section. Also small variations in film sensitivity and small batch differences in isotope activities have an affect. The variations in the overall optical density between sections which result from these sources of error, can confound data from regional measurements. The standardization procedure used is summarized in equation.2.

$$2. \frac{\text{Binding to region}}{\text{Overall binding to whole section}} \times \text{mean overall binding to all whole sections on film.}$$

6

Data analysis

The data presented in chapters 3 - 6 was processed using the following software for the Macintosh: Microsoft Excel 4.0 (© 1985-92 Microsoft corporation), Biosoft EBDA/LIGAND (© 1985 G.A.McPherson), Cricket Graph 1.3 (© 1986/87/88 Cricket software), Cricket Graph III, version 1.0 (© 1992 Computer Associates International, inc).

Data from the behavioural pharmacology experiments presented in chapters 4 and 6 were necessarily non-parametric and were, therefore, analysed statistically using 2x2 χ^2 tests. These were performed using a specially programmed Microsoft Excel spread sheet*. The specific binding data presented in chapters 3 and 5, as well as the kinetic parameters of binding presented in chapters 5 and 6, was analysed statistically using two way analysis of variance (ANOVA) with a split plot design, followed by Students t-tests. These were performed using the statistical capabilities of Excel 4.0. The kinetic parameters of equilibrium binding presented in chapters 5 and 6 were estimated from binding data using EBDA/LIGAND, originally, written by Munson and Rodbard (1980)¹⁴⁵, modified by McPherson (1985)¹³⁰ and based on the analytical methods of Weiland and Molinoff (1981)²²⁵. The basic principles underlying these methods are summarized in section 6.1.

* programmed by Maria Gullinello, Open University.

6.1

The principles of equilibrium binding kinetics.

Radioligands can be used in assays, such as those described in chapters 3, 5 and 6, to characterize the properties of a binding reaction between a ligand (eg, a neurotransmitter) and its receptors. As stated at the end of chapter 1, one of the major aims of this thesis was to determine whether passive avoidance training affects the concentration of receptors in a brain region, or their affinity. As in most other studies of this nature, therefore, these must be the most important properties to quantify. B_{max} is the parameter used to quantify the maximal binding of a ligand to its receptors, and therefore the concentration of receptors. It is expressed in terms of ligand concentration per unit mass of tissue or protein (eg, fmol.mg protein⁻¹). K_D is the equilibrium dissociation constant. It is defined by the ratio of bound to unbound ligand, quantified as the concentration of ligand at which the level of binding is half of B_{max} , and is inversely proportional to the affinity of a receptor for a ligand⁴⁶.

In the simplest model of a ligand-receptor interaction, it is assumed that a homogeneous univalent species of ligand binds to a single, non-interacting, population of binding sites. This is, of course, an equilibrium reaction which obeys simple mass action principles, and can be described by equation 3. This is a second order rate equation where L is unbound ligand, R is free receptor, LR is the ligand-receptor complex, k_1 and k_{-1} are the kinetic rate constants for association and dissociation respectively²²⁵.



Where the time course of association for this type of reaction can be determined, k_1 can be calculated using an integrated transformation of equation 3, ie, the integrated second order rate equation (see Weiland and Molinoff, 1981)²²⁵. k_{-1} can be similarly calculated from the time course of dissociation. The equilibrium dissociation constant is related to the kinetic rate constants by equation 4.

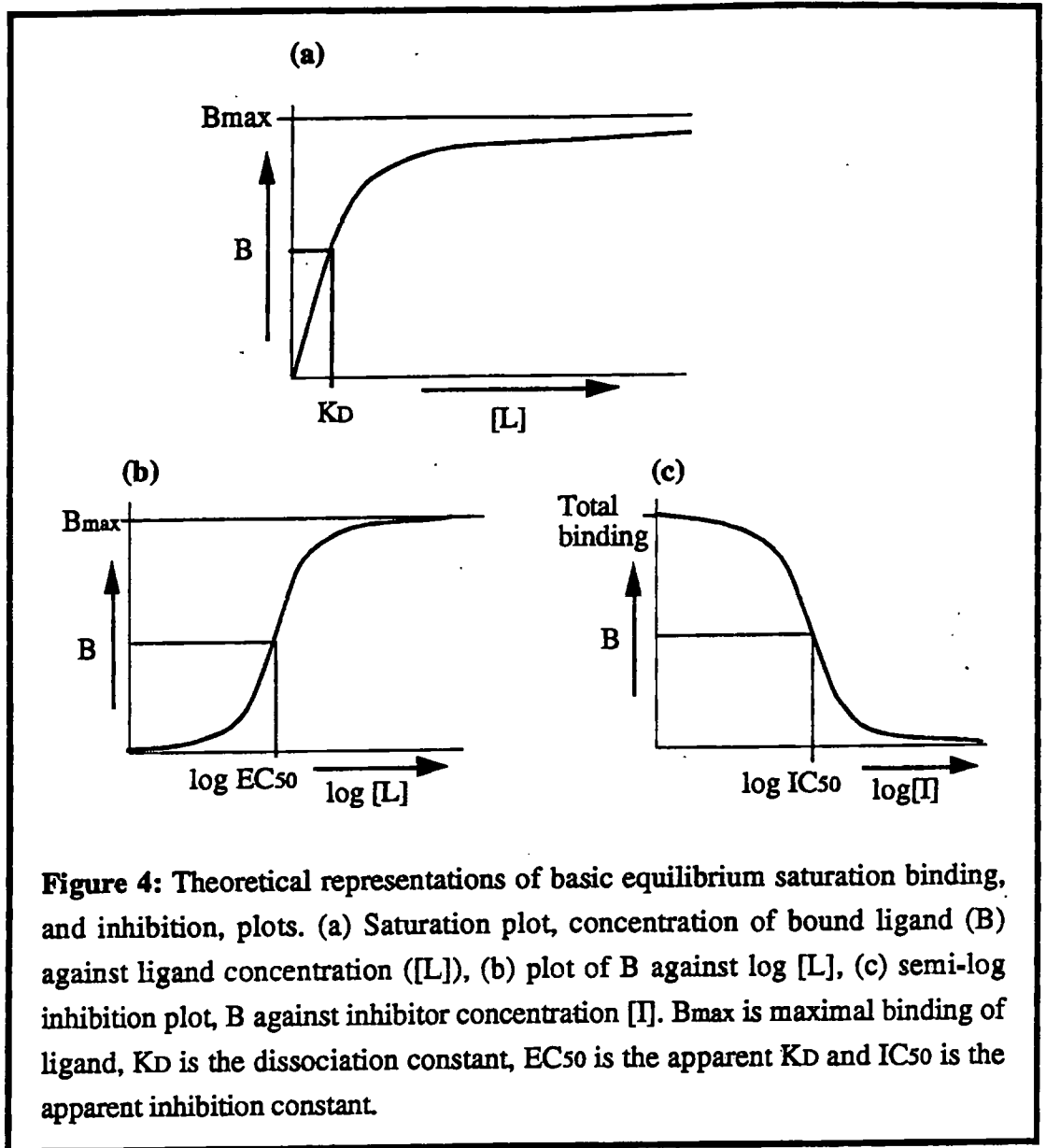
$$4. \quad K_D = \frac{k_{-1}}{k_1}$$

It is usually more convenient to analyze this type of reaction using equilibrium saturation binding data, where the concentration of receptors ($[R]$) is assumed to be constant and the ligand concentration ($[L]$) is varied. Here $[R]$ is equivalent to B_{\max} and the concentration of ligand bound (B) is equivalent to the concentration of the ligand-receptor complex at equilibrium for each $[L]$ ⁴⁶. Equation 5 is a generalization for this type of system.

5.
$$B = \frac{B_{\max} [L]}{K_D + [L]}$$

Equilibrium saturation binding analysis of this type was first used in 1903 by the French chemist Victor Henri, to characterize the initial rates of catalysed reactions¹⁰⁹. Hill (1910)¹⁰⁹ used a similar analysis to describe the cooperativity of oxygen-haemoglobin interactions. Perhaps the best known example, is that of Michaelis and Menten (1913)¹⁰⁹ who formulated an equation, similar to equation 5, to characterize enzyme catalysed reactions.

A plot of B against $[L]$ shows that equation 5 is a rectangular hyperbolic function similar to the Langmuir isotherm for the competitive adsorption of gases on a surface (1916)¹⁸. The curve flattens off as B approaches the maximum number of receptors, ie, B_{\max} . A theoretical saturation curve, illustrating the definitions of B_{\max} and K_D , is shown in figure 4 (a). It is usually very difficult to estimate B_{\max} and K_D values from this type of graph, as saturating concentrations of ligand are required. In some cases the estimation of these values can be made simpler by plotting B against $\log [L]$. Here, a sigmoid curve results, from which B_{\max} can be more readily estimated (figure 4, b). This type of graph, however, still requires saturating ligand concentrations and can only provide a relatively rough estimation of binding parameters, therefore, in most cases only the apparent equilibrium dissociation constant (EC_{50}) is estimable.



Where a direct analysis of radioligand binding such as that described above is not possible, an indirect analysis, where the ligand of interest is used as a competitive inhibitor (or more correctly, displacer) of a radioligand, can be performed. Here the binding parameters of the inhibitor can be characterized using a plot such as that shown in figure 4 (c), where ligand binding (sometimes expressed as % of total, or specific, binding in this type of study) is plotted against the log of inhibitor concentration ($\log [I]$). The inhibitor concentration at which ligand binding is equal to 50 % of the total, is defined as the apparent inhibition constant (IC_{50}). This is related to the true equilibrium inhibition constant (K_I) and to the K_D of the inhibitor for its receptors by the Cheng-Prussoff equation (equation 6)^{46,225}.

6.
$$K_I = \frac{IC_{50}}{1 + [L] / K_D}$$

Where the concentration of the ligand, being inhibited, is much less than the K_D of its receptors, the IC_{50} is equivalent to the true K_I . Deviation of IC_{50} from K_I becomes greater as $[L]$ is increased.

The two most useful methods of equilibrium saturation binding analysis, and those that were most extensively used to analyse the data in this thesis, are Scatchard and Hill analyses²²⁵. Scatchard analysis is based on a transformation of equation 5 which allows equilibrium saturation binding data, obeying simple second order kinetics, to be plotted as a straight line with a negative slope. Equation 7 is the Scatchard equation²²⁵.

7.
$$\frac{B}{F} = \frac{-B}{K_D} + \frac{B_{max}}{K_D}$$

B (the concentration of bound ligand) divided by F (the concentration of free ligand) is plotted against B (figure 5, a). Binding parameters can be estimated relatively accurately from this type of plot, whether saturating concentrations of ligand or inhibitor are used or not. The x intercept is B_{max} , the slope is equal to $-1/K_D$ and the y intercept is equal to B_{max}/K_D ^{46,225}. In experimental designs where the ligand of interest is used to displace a radioligand, specific binding of displacer can be defined as the concentration of radioligand displaced, ie, the data is treated as displacer sensitive radioligand (DSR) binding. Here, in a Scatchard plot of B/F against B , B is the concentration of DSR bound and F is the concentration of free displacer^{98,130}.

Often, ligand-receptor interactions are more complex than has been described so far (ie, they do not follow simple second order kinetics). In this case a Scatchard plot will deviate from linearity. A curvilinear Scatchard plot, concave upwards such as that shown in figure 5 (b), indicates lower binding affinity at higher ligand concentrations and can result from any of a number of factors. The most common of these are heterogeneity of binding

sites or ligand, negative cooperativity* between binding sites or a two step/three component binding reaction. Another possible explanation, and a common error, is that some non-specific binding could be mistakenly included in the plotted data, so care must be taken to avoid this. In order to discriminate between these possibilities a detailed kinetic and statistical analysis is necessary, although a prior knowledge of the receptors mechanism of action can help in many cases. A Scatchard plot which is concave downwards indicates lower binding affinity at lower ligand concentrations, and implies positive cooperativity* of binding.

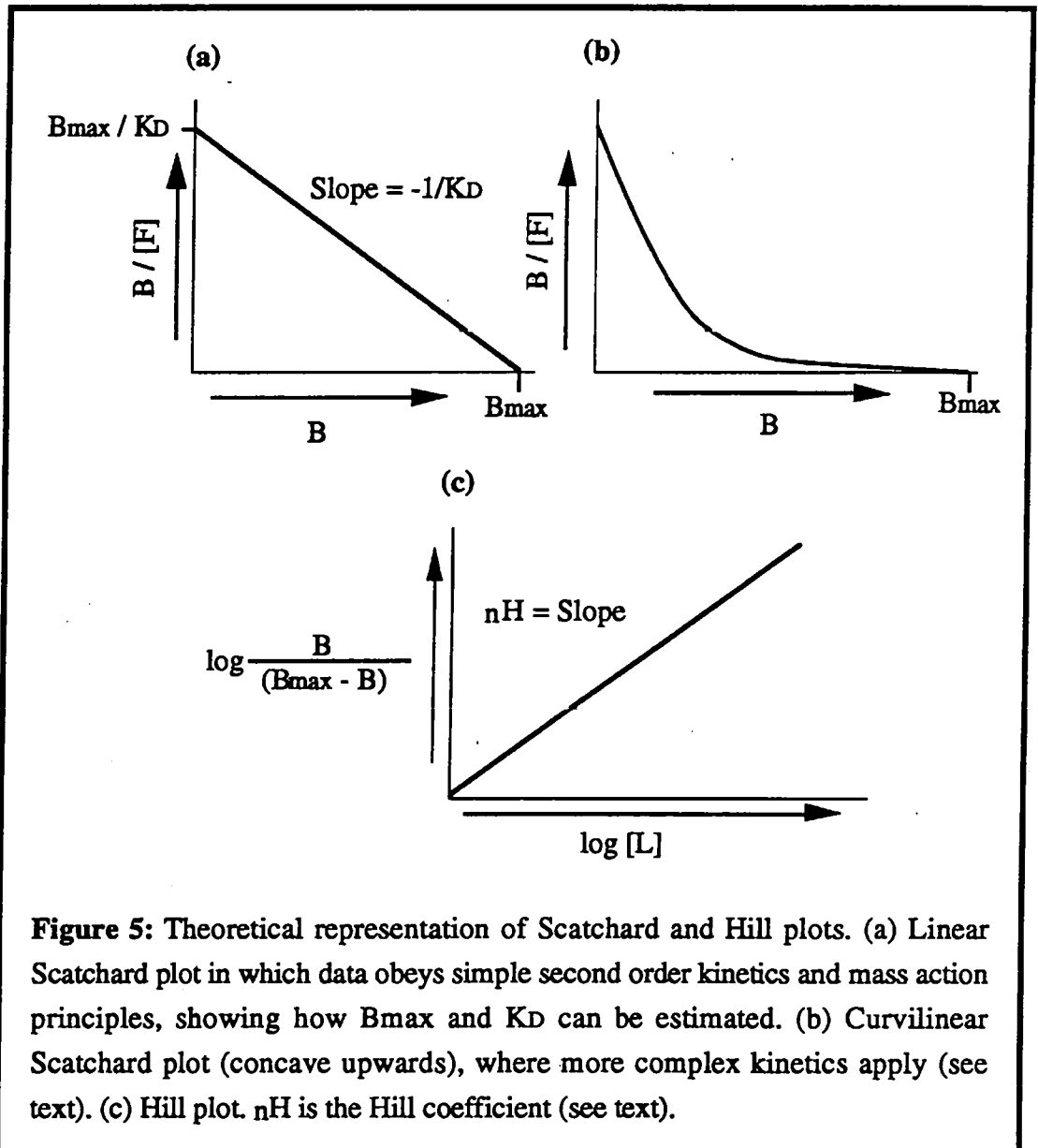
It is often difficult to show whether a straight line, indicating a simple one binding site model, or a non-linear curve, indicating a more complex interaction, should be fitted to the data points on a Scatchard plot. A statistical analysis such as that performed by the LIGAND software package^{130,145} can indicate which is the better fit. In order to show whether either cooperativity or binding site heterogeneity is involved, however, a Hill analysis of the same data is required. This is based on a logarithmic transformation of the Hill equation^{46,225} (equation 8).

8.
$$\log \frac{B}{(B_{\max} - B)} = n \log [L] - \log K_D$$

Hill analysis requires a knowledge of the concentration of binding sites, ie, B_{\max} . This allows data to be plotted as a straight line with a positive slope, $\log(B/B_{\max} - B)$ against $\log [L]$ (figure 5, c), whether the same data plots as a linear Scatchard plot or not. The slope of a Hill plot is equal to the Hill coefficient (n_H). One problem with Hill plots is that they tend to deviate from linearity at the extremes. Only the linear central portion of the plot is, therefore, equated with n_H . Where second order kinetics apply to a binding interaction, ie, where a Scatchard plot is linear, n_H will equal one. n_H of one and a linear Scatchard plot, however, does not necessarily prove that binding is a simple bimolecular interaction, as more complex interactions may also follow mass action principles. This requires a more detailed kinetic analysis. Where n_H is significantly less than one, the situation which results

* Negative cooperativity is where binding to a receptor site decreases the affinity of additional sites on the same protein. In Positive cooperativity binding results in increased affinity of additional sites⁴⁶.

in a concave upward Scatchard plot, ie, Binding site heterogeneity, negative cooperativity or two step/three component binding, is indicated. nH of significantly greater than one indicates positive cooperativity²²⁵.



Where two step/three component binding can be ruled out, nH also indicates either the number of binding sites involved in cooperativity, or the number of heterogeneous binding sites present in the tissue under examination. Where nH is less than one the fraction indicates the number of binding sites, and where nH is greater than one each whole integer indicates the number of binding sites (eg, nH of 0.5 or 2 indicates two binding sites, nH of

0.25 or 4 indicates four binding sites)⁴⁶. Where nH is a fraction of one which lies between those required for a particular number of binding sites (eg, 0.75) more than one factor, ie, all or any two of binding site heterogeneity, negative cooperativity or two step/three component binding, could be acting on the kinetics. In this case a careful statistical analysis is necessary to find whether nH is significantly different from a relevant fraction, eg, 1, 0.5, etc.

As previously mentioned, analysis of equilibrium binding data presented in this thesis was performed using EBDA/LIGAND. EBDA processes binding data by calculating the mean "bound" and "bound / free" values for Scatchard analysis, and the appropriate values for Hill analysis, then performs initial Scatchard and Hill analysis using linear regression in a single binding site model and gives initial estimates of the association (K_A) and dissociation (K_D) constants, in addition to a maximum binding value (B_{max}) and the Hill coefficient (nH). An initial inhibition analysis, in which an initial estimate of IC_{50} is calculated, can also be performed in EBDA. The resulting data can then be saved as a file suitable for analysis in LIGAND. LIGAND is an iterative curve fitting program which does not use regression, but modifies initial estimates of K_A and B_{max} to produce the best fit using the binding isotherm, and calculates final K_D and B_{max} values from the fit (one value for each parameter with a single site model, or 2 for each parameter with a two site model). It can also statistically compare a linear fit (single site model) with a non-linear fit (two site model) indicating which is the most appropriate. The major advantages of using this software to perform equilibrium binding analysis, over other methods, are that it uses an exact mathematical model of ligand binding which avoids possible biases, it uses a statistically valid and appropriately weighted curve fitting algorithm, and it corrects for any erroneous non-specific binding which may influence the results^{130,145}.

Chapter 3

Binding characteristics of L-glutamate receptors in the chick brain

7

Binding characteristics: preliminary studies.

Before undertaking the main body of work for this thesis, it was necessary to perform a number of preliminary experiments to investigate the binding properties of L-glutamate receptors in whole chick brain sections. The main purpose of this was to determine whether the conditions used were appropriate for the main body of autoradiographic work described in later chapters. The experiments reported in this section were designed to be performed quickly and easily in order to gain a general idea of the equilibrium binding properties of L-glutamate receptors. Rigorous statistical analyses were not possible due to the low number of chicks used in each experiment (normally 3), so the results obtained should only be regarded as approximations. The experiments performed involved the radioligand binding techniques and kinetic analysis described in chapter 2. Bound radioligand activity was measured using liquid scintillation counting as described in chapter 2 section 5.3.4.

7.1

Equilibrium binding kinetics of L-glutamate, its agonists and antagonists in whole chick brain sections.

Preliminary experiments 1 and 2 were designed to investigate the general properties of L-glutamate binding in the chick brain, its displacement by the competitive agonist NMDA and the competitive antagonist CPP, and the effect of the NMDA receptor co-agonist glycine on these properties. In preliminary experiments 3 and 4 the properties of [^3H]MK-801

binding to NMDA receptors and the those of [^3H]AMPA binding to non-NMDA receptors were investigated.

7.1.1: Experiment 1: Investigation of the inhibition of [^3H]L-glutamate binding to coronal sections from two forebrain locations of a day old, chick

The main purpose of this experiment was to determine the L-glutamate concentration at which its receptors become saturated. A secondary aim was to test whether there are any differences in the overall binding properties of L-glutamate receptors in whole sections cut from two different locations in a single chick brain (anterior and intermediate- see chapter 1, figure 3), ie, to show whether binding data from different locations can be meaningfully compared. A concentration of [^3H]L-glutamate (100 nmol l^{-1}) with varying concentrations of "cold" L-glutamate prepared using 50% serial dilutions, were applied to triplicate sections from both anterior and intermediate forebrain locations of a single day old chick. IC_{50} values were determined using EBDA/LIGAND, where % specific binding was plotted against $\log(\text{displacer concentration})$ (See section 6.1).

Procedure

A single day old chick was killed. Its brain was dissected out, divided into portions, and stored at -80°C , as described in chapter 2 section 5.3.1. When the brain was taken out of storage, the forebrain portion was placed in a cryostat where its temperature was allowed to equilibrate to -20°C . 50 consecutive coronal sections ($10\text{ }\mu\text{m}$ thick) were cut from the anterior part of the forebrain, and 50 more were cut from the intermediate part of the forebrain (see chapter 1 figure 3) and thaw mounted on cover slips. Sections were placed in numbered racks, dried in a cold air stream and stored at -20°C . When the sections were removed from storage they were allowed to come to room temperature. The 42 sections of best quality were selected from each batch of 50 and again placed in numbered racks. This gave 14 anterior triplicates and 14 intermediate triplicates.

Sections were pre-incubated for one hour at 4°C in TCB, then dip washed in distilled water and dried in a cold air stream. 13 concentrations of L-glutamate solution, in TCB (350µl volumes), were prepared by 50% serial dilution (top concentration 1 mmol l⁻¹). 350 µl of 200 nmol l⁻¹ [³H]L-glutamate (in TCB) was then added to each, and to 350µl of TCB for total binding, giving 14 solutions each containing 100 nmol l⁻¹ [³H]L-glutamate and with L-glutamate concentrations ranging from 500 µmol l⁻¹ to zero (see table 2 for [L-glutamate] in all solutions).

Sections were arranged in triplicates on aluminium sheets (on ice), with anterior and intermediate sections on separate sheets. Each of the 14 solutions were applied as a 100µl drop to completely cover each section of one anterior and one intermediate triplicate. Sections were incubated for 20 minutes on ice (approximately 4°C). Excess solution was removed from each coverslip and sections were washed three times in ice cold TCB (10 seconds for each), then dip washed in distilled water and dried in a cold air stream.

When dry, sections were examined, to make sure that no significant deterioration had occurred. Three intermediate sections had deteriorated badly. Five anterior sections, including one complete triplicate, had also deteriorated. these were all discarded and are indicated in table 2. Measurements of [³H]L-glutamate binding to sections were made using liquid scintillation counting (as described in chapter 2 section 5.3.4). Each section on its coverslip, was broken into a scintillation vial and 8ml of scintillant was added to each vial. The vials were then numbered and placed in the scintillation counter. In addition three scintillation vials, each containing 25 µl of the [³H]L-glutamate solution used, were treated in the same way as the others, so that an accurate value of [³H]L-glutamate concentration could be determined.

Results

The accurate concentration of the sample was calculated using the mean activity of 25 µl of [³H]L-glutamate solution and the sample's specific activity (s.a) was calculated as described in chapter 2 section 5.3.4. This was found to be 125.7 nmol l⁻¹. s.a for the sample was 93.02 dpm/fmol.

The activities of the triplicates to which the highest L-glutamate concentration (500 $\mu\text{mol l}^{-1}$) was applied were considered to be NSB. This value was 64.81 ± 12.96 dpm for anterior sections and 68.72 ± 8.79 dpm for intermediate sections. These values were taken to represent zero specific binding or 100 % inhibition, and were subtracted from all other activity values during processing in EBDA to give specific binding values. Mean % specific binding and % inhibition of [^3H]L-glutamate binding for each [displacer], with standard errors of the mean, are shown in table 2. The total binding value represents 100% specific binding and zero inhibition.

Saturation plots of % inhibition against [displacer], and semi-log inhibition plots of % specific binding against log[displacer] generated by EBDA/LIGAND, for both anterior and intermediate sections are shown in figure 6. The IC_{50} values calculated by EBDA/LIGAND were: anterior $\text{IC}_{50} = 1.1 \pm 0.49$ $\mu\text{mol l}^{-1}$, intermediate $\text{IC}_{50} = 0.66 \pm 0.21$ $\mu\text{mol l}^{-1}$.

Table 2: Results from preliminary experiment 1 (see text). Values are means from triplicate sections taken from a single chick \pm standard error of the mean. * indicates sections discarded due to deterioration during processing.

[L-glu] $\mu\text{mol l}^{-1}$	log [L-glu] $\mu\text{mol l}^{-1}$	% specific binding (anterior)	% inhibition (anterior)	% specific binding (intermed)	% inhibition (intermed)
500	2.7	0 ± 4.4	100 ± 4.4	0 ± 2.0	100 ± 2.0
250	2.4	4.5 ± 1.8	95.5 ± 1.8	0.8 ± 1.5	99.2 ± 1.5
125	2.1	1.6 ± 1.9	98.4 ± 1.9	1.6 ± 1.6	98.4 ± 1.6
62.5	1.8	6.7 ± 2.4	93.3 ± 2.4	4.8 ± 1.8	95.2 ± 1.8
31.25	1.5	13.3 ± 2.0	86.7 ± 2.0	10.5 ± 1.5	89.5 ± 1.5
15.63	1.2	11.4 ± 2.1	88.6 ± 2.1	13.2 ± 6.5	86.8 ± 6.5
7.81	0.9	21.1 ± 3.8	78.9 ± 3.8	17.7 ± 6.7	82.3 ± 6.7
3.91	0.6	*	*	21.6 ± 2.8	78.4 ± 2.8
1.95	0.3	41.6 ± 7.2	58.4 ± 7.2	21.9 ± 1.5	78.1 ± 1.5
0.977	-0.01	43.4 ± 6.7	56.6 ± 6.7	28.5 ± 3.0	71.5 ± 3.0
0.488	-0.3	57.8 ± 3.2	42.2 ± 3.2	64.0 ± 4.8	36.0 ± 4.8
0.244	-0.6	68.7 ± 3.3	31.3 ± 3.3	64.7 ± 4.5	35.3 ± 4.5
0.122	-0.9	87.8 ± 3.0	12.2 ± 3.0	80.7 ± 3.3	19.3 ± 3.3
0		100 ± 3.7	0 ± 3.7	100 ± 0.7	0 ± 0.7

Discussion

Figure 6(c) and (d) show a large degree of similarity between the semi-log displacement curves for anterior and intermediate sections. The values of IC_{50} from the two curves do not differ, within margins of error. This indicates that the binding properties of L-glutamate receptors in the two forebrain locations do not differ appreciably.

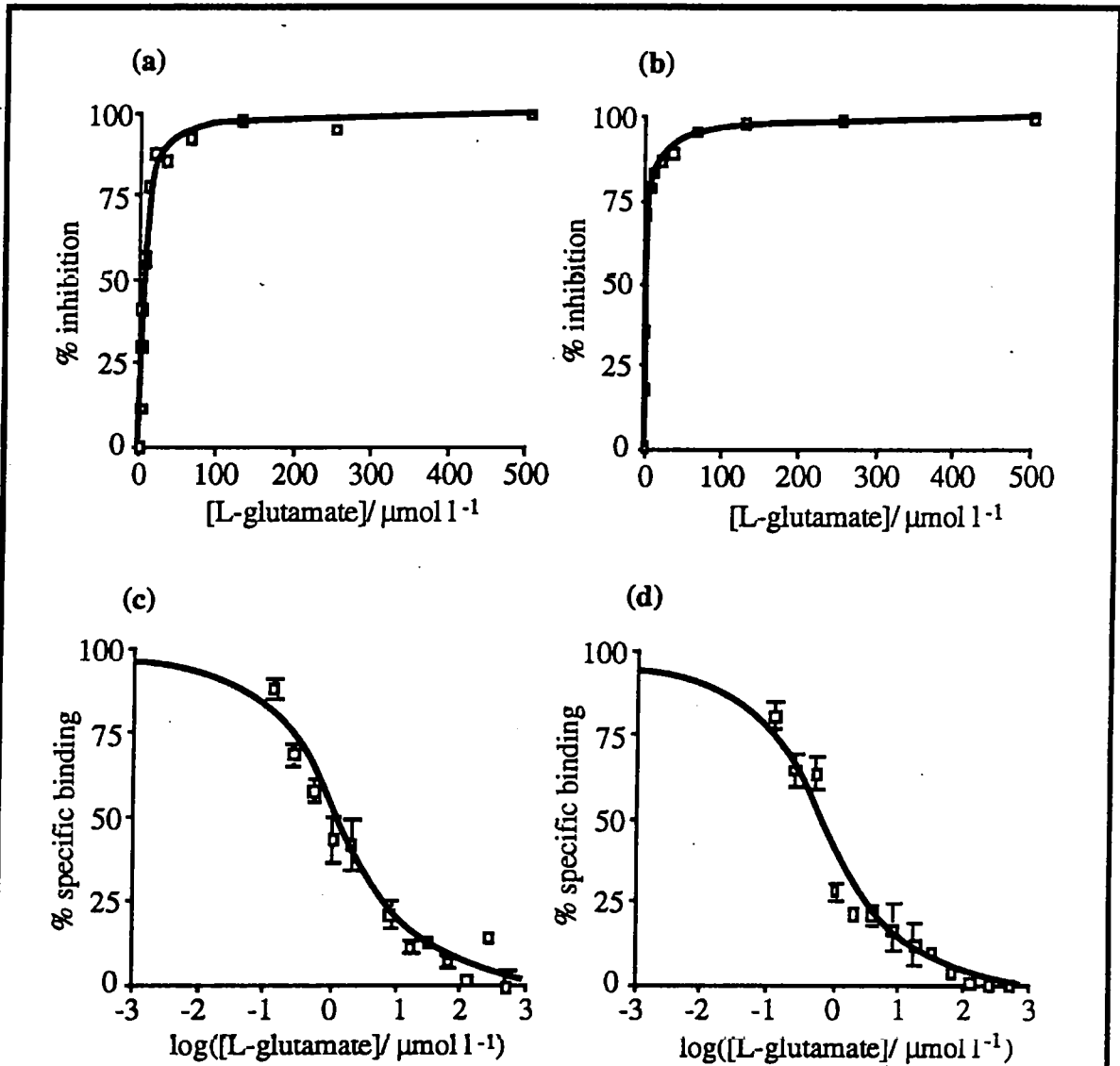


Figure 6: Saturation and semi-log inhibition plots of binding data from preliminary experiment 1, inhibition of $[^3\text{H}]$ L-glutamate binding by L-glutamate. The data was processed and the plots generated using EBDA/LIGAND. (a) Saturation plot for anterior sections, (b) saturation plot for intermediate sections, (c) semi-log inhibition plot for anterior sections, (d) semi-log inhibition plot for intermediate sections. Values are the means of triplicate sections taken from a single chick brain. Error bars represent standard error of the mean.

It can be seen from figure 6 (a and b) that the saturation curve for this system flattens off at a displacer concentration of about $100 \mu\text{mol l}^{-1}$ and at a point close to 100 %. This indicates that almost all of the bound [^3H]L-glutamate can be displaced using this concentration, and it can be equated with the concentration of L-glutamate at which its binding to the receptors approaches saturation.

7.1.2: Experiment 2: Investigation of the inhibition of [^3H]L-glutamate binding to coronal sections from day old chick forebrain by L-glutamate, NMDA and CPP.

This experiment had two major aims. The first of these was to determine the effectiveness of the competitive agonist NMDA and the competitive antagonist CPP, in displacing [^3H]L-glutamate binding from chick forebrain sections, by measuring the IC_{50} of the three displacers and the % inhibition of NMDA and CPP with respect to L-glutamate. The second aim was to determine the effect of the NMDA receptor co-agonist glycine on these parameters, which would determine whether glycine should be present as a potentiator of [^3H]L-glutamate binding in further experiments.

Procedure

Forebrains from three day old chicks were prepared as described in chapter 2 (section 5.3.1). 174 $10 \mu\text{m}$ thick coronal sections were cut from each of these, thaw mounted on coverslips, dried and stored, following the procedure described in chapter 2 (section 5.2.2). Prior to pre-incubation sections were allowed to come to room temperature. 162 sections were selected from each batch of 174 for two separate assays (ie, 81 per assay). In each assay, three sets of triplicate sections (1 from each brain) for total [^3H]L-glutamate binding, plus three sets of duplicates (again one from each brain) for each of 13 displacer (L-glutamate, NMDA or CPP) concentrations were used (ie, a total of 243 sections). These were divided appropriately between 14 numbered racks.

In each assay, sections were pre-incubated for one hour at 4°C in TCB, then dip washed in distilled water and dried in a cold air stream. In the first assay 13 concentrations

each of L-glutamate, NMDA and CPP (350 μl volumes) were prepared by 50 % serial dilution (maximum concentration 200 $\mu\text{mol l}^{-1}$). 350 μl of 200 nmol l^{-1} [^3H]L-glutamate (in TCB) was then added to each. 500 μl of 200 nmol l^{-1} [^3H]L-glutamate was also added to 500 μl of TCB for total binding. This gave 40 solutions each containing 100 nmol l^{-1} [^3H]L-glutamate, 39 of which contained serial dilutions of displacer (13 L-glutamate, 13 NMDA and 13 CPP) ranging from 100 to 0.024 $\mu\text{mol l}^{-1}$, with the total binding solution acting as zero displacer for all three. The three sets of 13 serial dilutions for the second assay were prepared in an identical fashion, except that 250 μl volumes were prepared and the top displacer concentration was 300 $\mu\text{mol l}^{-1}$. To each of these 250 μl of 1.5 mmol l^{-1} glycine (in TCB) was added, followed by 250 μl of 300 nmol l^{-1} of [^3H]L-glutamate, to give the same range of displacer and [^3H]L-glutamate concentrations as in the first assay, but in the presence of 500 $\mu\text{mol l}^{-1}$ glycine. The total binding solution was made up similarly, by adding 320 μl of 300 nmol l^{-1} [^3H]L-glutamate and 320 μl of 1.5 mmol l^{-1} glycine to 320 μl of TCB.

In both assays sections were arranged appropriately on aluminium sheets (on ice) and incubations were performed as described in experiment 1, but with total binding solutions and each of the three sets of displacer serial dilutions. No significant damage to sections was observed in either session. Binding of [^3H]L-glutamate to sections and its accurate concentration were measured using liquid scintillation counting as described previously (Chapter 2 section 5.3.4 and experiment 1).

Results

The accurate concentrations of [^3H]L-glutamate used in each assay (calculated as described in experiment 1) were found to be 80.16 nmol l^{-1} in the first and 73.64 nmol l^{-1} in second. s.a for the sample was 108.78 dpm/fmol.

As in experiment 1, the activities of the triplicates to which the highest L-glutamate concentration (100 $\mu\text{mol l}^{-1}$) was applied were considered to be NSB. These values were 71.67 ± 7.3 dpm in the first assay and 55.43 ± 6.81 dpm in the second (with glycine), and

were taken to represent zero specific binding (100% displacement) for all three displacers. They were subtracted from all other activity values during data processing in EBDA to give specific binding values. Mean % specific binding was plotted against log[displacer] for each of L-glutamate, NMDA and CPP, both in the absence and presence of glycine (Figure 7).

% inhibition of [^3H]L-glutamate by $100\ \mu\text{mol l}^{-1}$ NMDA (compared to 100 % inhibition by $100\ \mu\text{mol l}^{-1}$ L-glutamate) was $64.25 \pm 5.78\%$ ($61.94 \pm 3.08\%$ in the presence of glycine) and by $100\ \mu\text{mol l}^{-1}$ CPP was $70.12 \pm 10.93\%$ ($62.01 \pm 1.72\%$ in the presence of glycine). These values were calculated from binding data and not from the graphs shown in figure 7.

Statistical analysis by LIGAND indicated that a two component model fitted the data significantly better than a one component model for L-glutamate displacement of [^3H]L-glutamate, both in the absence and presence of glycine. ANOVA: $F_{24,23} = 2.2$, $p = 0.032$, without glycine, $F_{24,23} = 2.48$, $p = 0.017$ with glycine. Two component curves were fitted to the data and are shown in Figure 7 (a) and (b). IC_{50} values from these are as follows: In the absence of glycine, $\text{IC}_{50} = 23 \pm 4.2\ \text{nmol l}^{-1}$ (high affinity component), $\text{IC}_{50} = 2.67\ \mu\text{mol l}^{-1}$ (low affinity component), In the presence of glycine $\text{IC}_{50} = 43 \pm 14\ \text{nmol l}^{-1}$ (high affinity component), $\text{IC}_{50} = 8.17 \pm 1.99\ \mu\text{mol l}^{-1}$ (low affinity component).

From visual inspection of the data presented in Figure 7 (a) and (b), the distribution of data for NMDA displacement in the presence of glycine, and CPP displacement in the absence of glycine, of [^3H]L-glutamate binding appeared to have two components. This was not borne out by statistical analysis, however, although p for two compared to one component inhibition for the former was fairly close to significance. A two component fit was not a significantly better than a one component fit for any of the NMDA or CPP displacement data. ANOVA: $F_{24,23} = 0.73$, $p = 0.78$ for NMDA in the absence of glycine, $F_{24,23} = 1.91$, $p = 0.063$ in the presence of glycine, $F_{24,23} = 1.12$, $p = 0.39$ for CPP in the absence of glycine, $F_{24,23} = 1.78$, $p = 0.085$ in the presence of glycine. Single component curves were fitted to the data and are shown in figure 7 (a) and (b).

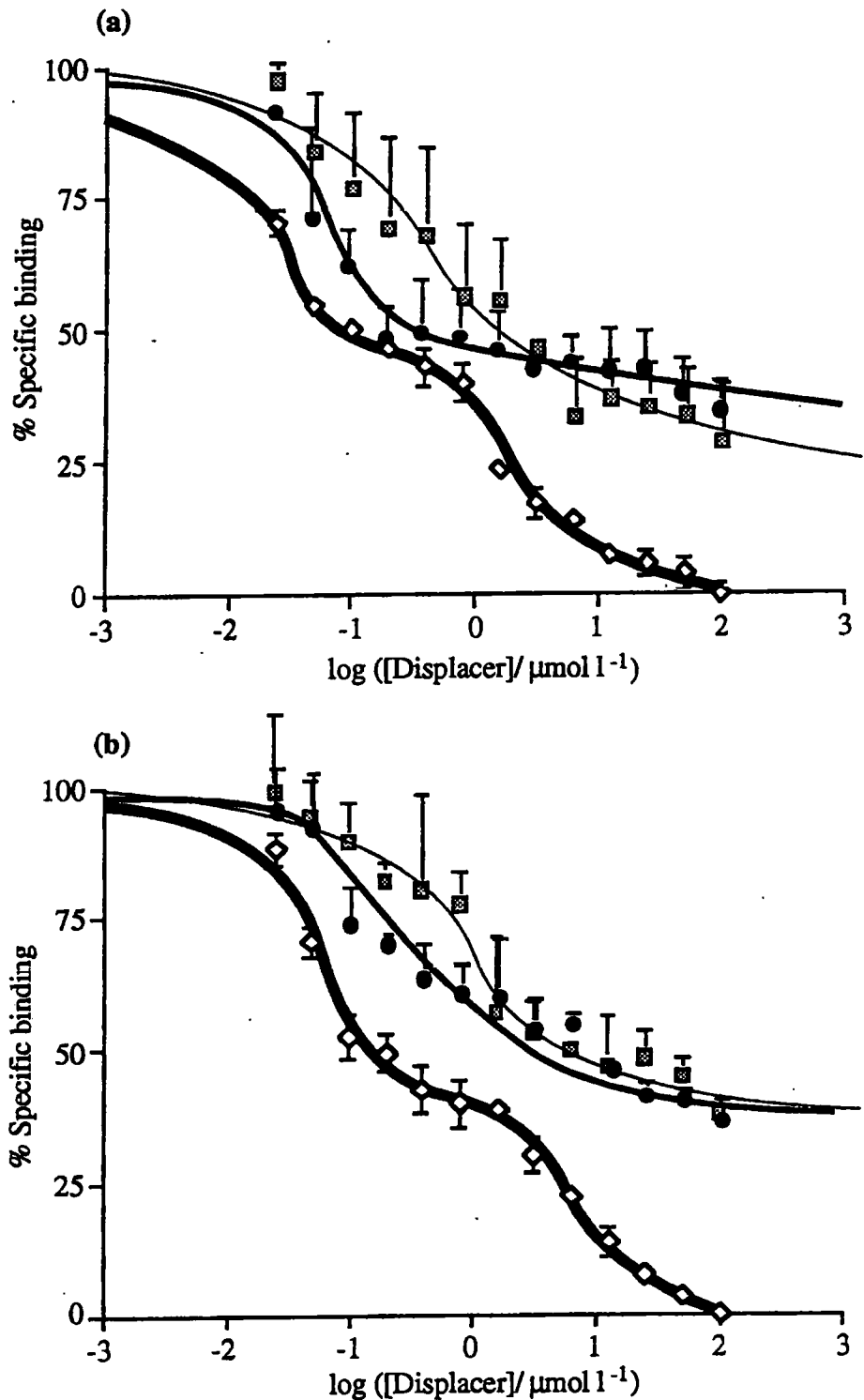


Figure 7: Semi log inhibition plots, from data processed in EBDA/LIGAND, showing displacement of $[^3\text{H}]$ L-glutamate by L-glutamate (\diamond — \diamond), NMDA (\bullet — \bullet) and CPP (\boxtimes — \boxtimes), (a) in the absence of glycine, (b) in the presence of glycine. Error bars represent standard error of the mean, $n = 3$ for each data point with 2 replicates per chick, except for total binding (3 replicates per chick). See text for IC_{50} values.

IC₅₀ values for NMDA and CPP displacement are as follows: for displacement by NMDA in the absence of glycine IC₅₀ = 0.103 ± 0.037 μmol l⁻¹, for displacement by NMDA in the presence of glycine IC₅₀ = 0.3 ± 0.14 μmol l⁻¹, for displacement by CPP in the absence of glycine IC₅₀ = 0.56 ± 0.27 μmol l⁻¹, for displacement by CPP in the presence of glycine IC₅₀ = 1.07 ± 0.33 μmol l⁻¹.

Discussion

The % inhibition of [³H]L-glutamate, compared to 100% inhibition by L-glutamate is the same for NMDA and CPP in the presence and absence of glycine, within margins of error. This result suggests that high (close to saturating) concentrations of NMDA and CPP are equally effective in displacing [³H]L-glutamate. Both NMDA and CPP, however, only displace around two thirds of bound [³H]L-glutamate. This suggests that under the experimental conditions used, two thirds of the receptors to which [³H]L-glutamate bound were of the NMDA type. As can be seen from both of graphs (a) and (b) in figure 7, the high affinity component of the L-glutamate displacement curve also accounts for about two thirds of the [³H]L-glutamate displaced. Also the single component displacement curves for NMDA and CPP seem to be equivalent to the high affinity component of the L-glutamate curve. This suggests that the high affinity component of the L-glutamate curve accounts for displacement of [³H]L-glutamate from NMDA receptors. The low affinity component of the curve, in both graphs (a) and (b), could account for displacement from any combination of a number of different binding sites. These could include AMPA receptors, KA receptors, metabotropic receptors and possibly L-glutamate uptake sites. On first inspection of the NMDA and CPP data, it looked as though a two component model might also be appropriate. This, however, was not borne out statistically, probably due to the relatively high standard errors being associated with these data. So, further experiments are necessary to show whether more than one NMDA receptor subtype is present in the chick forebrain. Also, Scatchard and Hill analysis (as described in chapter 2 section 6.1) would probably give a clearer view of this. The displacement curves in Figure 7 (a) and (b) and their associated IC₅₀ values clearly indicate that the three displacers investigated have the following rank order of affinity for NMDA receptors: L-glutamate > NMDA > CPP. As only

three brains were used in this preliminary investigation, however, the n number was too low to allow a full statistical analysis of the differences between the IC₅₀ values.

It is clear from figure 7 (a) and (b) that the presence of glycine in the incubation medium had a number of effects upon the displacement of [³H]L-glutamate binding by the three displacers. The most obvious of these is that [³H]L-glutamate became more difficult to displace where glycine was present. The IC₅₀ values for the three displacers appeared higher when glycine was present (although this result would require further validation using a higher number of chicks), suggesting that glycine potentiated [³H]L-glutamate binding to NMDA receptors, at the expense of L-glutamate, NMDA and CPP. It seemed best, therefore, not to include glycine in the incubation mixture in experiments where such displacement is necessary. A less obvious effect of the presence of glycine is that it seemed to increase the probability that a two component curve fit would be an improvement over a one component model, for all three displacers.

There is an obvious discrepancy between the data presented here and that presented in experiment 1. This is that the curve for displacement of [³H]L-glutamate binding by L-glutamate in experiment 1 only has one component, whereas in this experiment the equivalent curve (without glycine) has two. The probable explanation for this is that in experiment 1 the lowest concentrations of the displacer (L-glutamate) were not sufficient to allow the high affinity component to be identified. The low affinity IC₅₀ value presented here is higher than those presented in experiment 1 by at least a factor of two. Also, as the three lowest displacer concentrations used here were lower than the K_D for [³H]L-glutamate binding to chick forebrain tissue (K_D = 0.1 μmol l⁻¹ ¹²⁵), whereas those used in experiment 1 were not, it is likely that the IC₅₀ values are closer to the true K_i than those presented in experiment 1.

7.1.3: Experiment P3: Investigation of [³H]MK-801 binding to NMDA receptors in coronal sections from day old chick forebrain.

MK-801, as discussed in chapter 1 section 2.1.2, is a highly selective non-competitive antagonist of NMDA receptors which binds to a site within the ion channel, blocking the flow of Ca²⁺ through activated receptors. The effectiveness of MK-801 as an antagonist, therefore, increases in proportion to the number of open NMDA receptor channels, ie, it is use dependant^{11,48,110}. This experiment was performed in two parts. In part (a) the use dependency of [³H]MK-801 binding was tested with a fixed concentration of [³H]MK-801 and various L-glutamate concentrations, and in part (b) a Scatchard analysis was performed using various concentrations of [³H]MK-801, in order to gain some preliminary knowledge of the equilibrium kinetics of MK-801 binding in chick brain sections.

Procedure

Forebrains from three day old chicks were prepared as described in chapter 2 (section 5.3.1). Sixty 10 µm thick coronal sections were cut from each of these, thaw mounted on coverslips, dried and stored, following the procedure described in chapter 2 (section 5.3.2). In both of parts (a) and (b), performed on consecutive days, 27 sections from each brain were selected and allowed to come to room temperature. This gave nine triplicate sections from each brain which were arranged appropriately in numbered racks and pre-incubated as described in preliminary experiments 1 and 2, but at room temperature (chapter 2, section 5.3.3).

In part (a) 7 concentrations of L-glutamate in TCB were prepared by 90 % serial dilutions (maximum concentration 1.1 mmol l⁻¹). 100 µl of 50 nmol l⁻¹ [³H]MK-801 was then added to 900 µl of each solution, 900 µl of TCB and 900 µl of solution for NSB, giving a final [³H]MK-801 concentration of 5 nmol l⁻¹ in each of nine solutions with final L-glutamate concentrations of 1000, 100, 10, 1, 0.1, 0.01, 0.001 and 0 µmol l⁻¹. The NSB solution was prepared by adding 450 µl of 220 µmol l⁻¹ MK-801 to 450 µl of 2.2 mmol l⁻¹

L-glutamate, then adding 100 μl of 50 nmol l^{-1} [^3H]MK-801. This gave final concentrations of 1 mmol l^{-1} L-glutamate, 100 $\mu\text{mol l}^{-1}$ MK-801 and 5 nmol l^{-1} [^3H]MK-801.

In part (b) 8 concentrations of [^3H]MK-801 in a fixed concentration of L-glutamate were prepared by initially adding 1 ml of 160 nmol l^{-1} [^3H]MK-801 to 1 ml of 20 $\mu\text{mol l}^{-1}$ L-glutamate (both in TCB), followed by 50% serial dilutions in 20 $\mu\text{mol l}^{-1}$ L-glutamate, giving a final concentration of 10 $\mu\text{mol l}^{-1}$ L-glutamate and a range of [^3H]MK-801 concentrations (top, approximately 80 nmol l^{-1}) in each 1 ml volume of solution. A solution for NSB was also prepared by adding 250 μl of 40 $\mu\text{mol l}^{-1}$ L-glutamate to 250 μl of 400 $\mu\text{mol l}^{-1}$ MK-801, then adding 500 μl of 160 nmol l^{-1} [^3H]MK-801.

In both parts of this experiment incubations were performed as described previously (Experiment P1 and chapter 2 section 5.3.3) except that the nine sets of triplicate sections from each of the three brains were arranged in an appropriate sequence on aluminium sheets in a humid chamber at room temperature. In both parts of the experiment 100 μl of each of the nine solutions was applied to each of nine sections, ie, one triplicate from each brain. Washing times were three \times 30 s in TCB plus a dip wash in distilled water to remove buffer salts. Sections did not suffer any significant damage during incubation. The accurate concentration of [^3H]MK-801 and its binding to sections were measured by liquid scintillation counting in both parts of the experiment, as previously described (chapter 2 section 5.3.4 and experiment 1).

Results

The accurate concentrations of [^3H]MK-801 used in this experiment (calculated as described in experiment 1) were found to be 5.35 nmol l^{-1} in part (a) and 83.4 nmol l^{-1} for the top concentration in part (b). s.a for the sample was 63.94 dpm/fmol. Data from part (a) were analysed by plotting % specific [^3H]MK-801 binding against log L-glutamate concentration (figure 8, a). 100 % specific [^3H]MK-801 binding was defined as the activity, in dpm, in the presence of the highest concentration of L-glutamate used (ie, 1 mmol l^{-1})

after subtraction of non-specific binding (49.47 ± 3.21 dpm). This value was 529.24 ± 14.18 dpm and all other values were taken as a percentage of it.

The % difference in [^3H]MK-801 bound, as a function of the increase in L-glutamate concentration from 0 to 1 mmol l^{-1} , was 48 ± 12.23 %. This represents a 92 % increase in [^3H]MK-801 binding. The % [^3H]MK-801 binding is the same, within margins of error, for L-glutamate concentrations between 0 and $0.1 \text{ } \mu\text{mol l}^{-1}$, and for those between 1 and $1000 \text{ } \mu\text{mol l}^{-1}$. As can be seen in figure 8 (a), however, there is a sharp increase in [^3H]MK-801 binding between 0.1 and $1 \text{ } \mu\text{mol l}^{-1}$ which accounts for most of the overall increase.

Data from part (b) were processed in EBDA/LIGAND where Scatchard and Hill analyses were performed (see figure 8, b for Scatchard plot). Again, the NSB value, 51.54 ± 1.82 dpm, was taken to represent zero specific binding and was subtracted from all other binding values during processing in EBDA/LIGAND. Equilibrium binding values calculated by EBDA/LIGAND were: $K_D = 6.36 \pm 0.28 \text{ nmol l}^{-1}$, $B_{\text{max}} = 1713.6 \pm 40.2 \text{ fmol.mg prot}^{-1}$, $n_H = 0.987 \pm 0.04$.

Discussion

The results shown in figure 8 (a) demonstrate the use dependency of MK-801 binding to NMDA receptor channels. As the concentration of L-glutamate was increased from zero to $0.1 \text{ } \mu\text{mol l}^{-1}$, there was little or no increase in [^3H]MK-801 binding, indicating that concentrations in this range are insufficient to enhance binding to any further NMDA receptors. Between the L-glutamate concentrations of 0.1 and $1 \text{ } \mu\text{mol l}^{-1}$, however, a sharp increase in [^3H]MK-801 binding occurred, which levelled off at concentrations higher than this. No further increase in [^3H]MK-801 binding occurred at L-glutamate concentrations up to 1 mmol l^{-1} . This indicates that at L-glutamate concentrations above $1 \text{ } \mu\text{mol l}^{-1}$, the maximum possible number of NMDA receptor channels were open and that the threshold of activation of NMDA receptors by L-glutamate is $\approx 0.1 \text{ } \mu\text{mol l}^{-1}$. It was decided that $10 \text{ } \mu\text{mol l}^{-1}$ of L-glutamate would be an appropriate concentration to potentiate [^3H]MK-801 binding in further experiments.

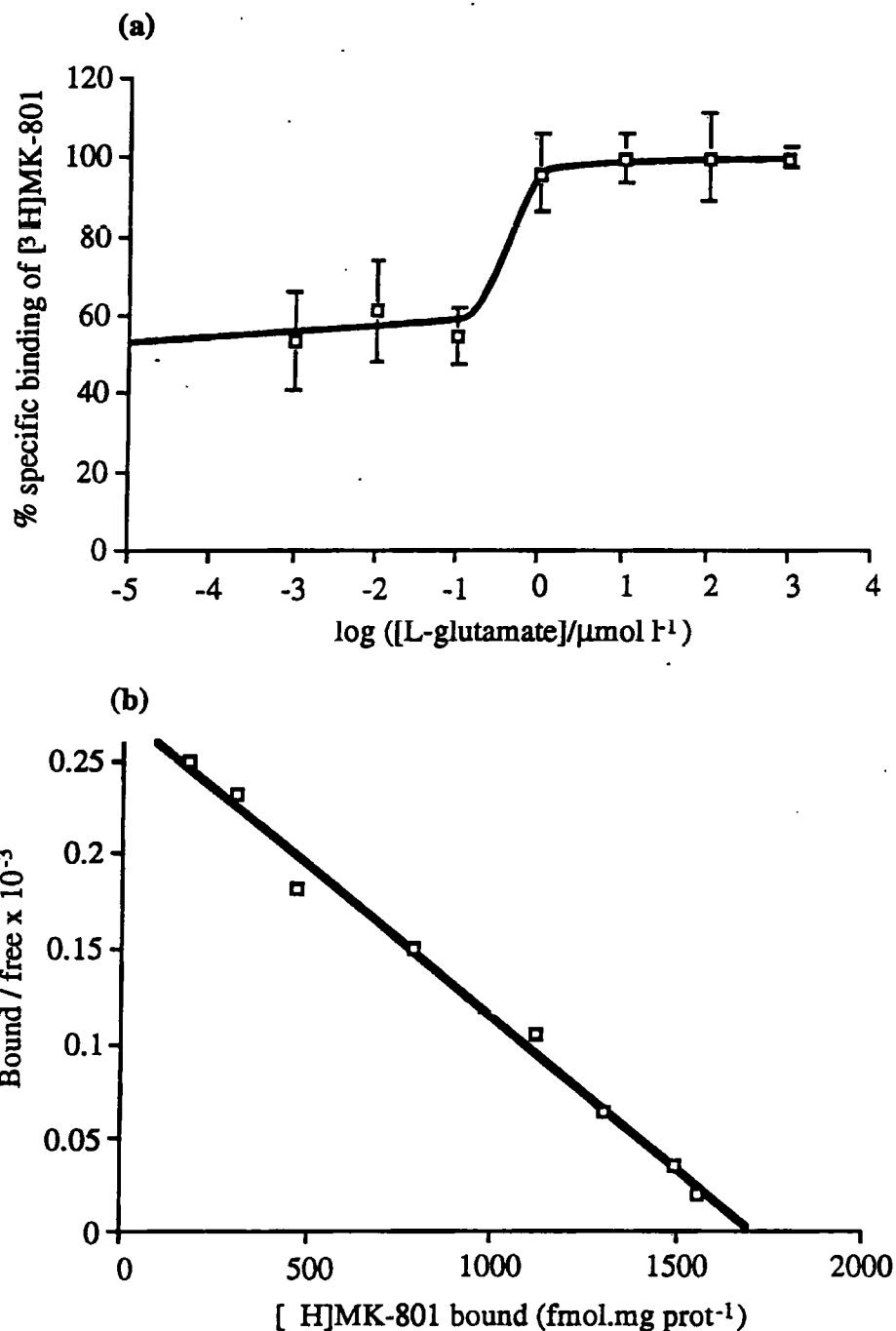


Figure 8: (a) Graph of % specific $[^3\text{H}]\text{MK-801}$ binding to chick brain sections against log L-glutamate concentration, demonstrating the use dependency of MK-801 binding to NMDA receptor channels (see text for details). Error bars represent standard error of the mean. (b) Scatchard plot for $[^3\text{H}]\text{MK-801}$ binding to chick brain sections, generated from data processed in EBDA/LIGAND (see text for details). $n = 3$ for both plots with three replicates per chick..

It is possible that an even greater number of NMDA receptors could have been opened and, therefore, a larger increase in MK-801 binding observed, if glycine had been present in the incubation medium in addition to L-glutamate. It has been shown in cultured chick neurons that glycine and NMDA together elicit a larger Ca^{2+} influx than NMDA alone²⁷. Such an experiment, however was not carried out due to shortage of time.

One seeming anomaly is that even where no L-glutamate has been added, [³H]MK-801 binds to more than 50 % of its binding sites. This would suggest that, under the conditions used, [³H]MK-801 has access to its binding site in many NMDA receptor channels without activation by L-glutamate. One explanation for this could be that, under these conditions, many NMDA receptors remain open even after endogenous L-glutamate and glycine have been washed out during pre-incubation. An alternative explanation could be that MK-801 binding is not as selective in the chick brain as was thought and that it binds to other proteins, in addition to NMDA receptors. This, however, seems to be precluded by the results of part (b).

The data from part (b) were plotted using a one site model in LIGAND, as a straight line Scatchard plot indicating the presence of a single class of binding sites for [³H]MK-801. Figure 8 (b) clearly shows no evidence for a two site model. This was supported by the Hill coefficient which approximated to 1 (0.987 ± 0.04). The K_D calculated by LIGAND at $6.36 \pm 0.28 \text{ nmol l}^{-1}$ seems to agree with that found by Kavanah, et al (1991)⁹⁹ who found a K_D of $5.3 \pm 0.5 \text{ nmol l}^{-1}$ using forebrain tissue from chicks aged 11 days old, particularly as this group have also shown that the K_D for [³H]MK-801 binding tends to decrease as chicks increase in age. In this previous study, however, some evidence in favour of a two site model was observed. The B_{max} of 1713.6 ± 40 found here, is also in broad agreement with the previous study. Although the maximum [³H]MK-801 concentration used was not saturating, the range of concentrations used were appropriate for Scatchard analysis and a similar range could be used for further experiments.

7.1.4: Experiment 4: Investigation of [^3H]AMPA binding to coronal sections from day old chick forebrain.

AMPA receptors, as discussed in chapter 1 section 2.1.1, are the most abundant class of excitatory receptor in the vertebrate brain^{72,165}. They are widely and relatively homogeneously distributed in the chick brain, but seem to be most abundant in the cerebellum and hippocampus^{84,231}. This experiment was designed to find the kinetic parameters of [^3H]AMPA binding to coronal sections from forebrains of untrained day old chicks. This involved binding a range of [^3H]AMPA concentrations to sections in the presence of SCN^- , which has been reported to potentiate binding of [^3H]AMPA to its receptors in rat brain membranes⁷², in order to decide whether the [^3H]AMPA concentration range and the SCN^- concentration used was appropriate for use in further autoradiographic experiments.

Procedure

Forebrains from three untrained day old chicks were prepared as described in chapter 2, section 5.3.1. 20 $10\ \mu\text{m}$ thick consecutive coronal sections were cut from each of these, thaw mounted on coverslips, dried and stored, following the procedure described in chapter 2, section 5.3.2. 11 sections from each brain were selected and allowed to come to room temperature, this gave 11 triplicates each comprising one section from each of the three brains which were arranged in appropriately numbered racks and pre-incubated as described in experiments 1 and 2. Ten concentrations of [^3H]AMPA were prepared in $100\ \text{mmol l}^{-1}$ KSCN solution (in TCB) by 50 % serial dilution (top concentration: $200\ \text{nmol l}^{-1}$, $400\ \mu\text{l}$ volumes). A solution for NSB was prepared by adding $200\ \mu\text{l}$ of $400\ \text{nmol l}^{-1}$ [^3H]AMPA to $200\ \mu\text{l}$ of $2\ \text{mmol l}^{-1}$ L-glutamate in $200\ \text{mmol l}^{-1}$ KSCN (in TCB). giving final concentrations of $200\ \text{nmol l}^{-1}$ [^3H]AMPA, $1\ \text{mmol l}^{-1}$ L-glutamate and $100\ \text{mmol l}^{-1}$ KSCN.

Incubations were performed as described previously (Experiment 1 and chapter 2 section 5.3.3) except that the washing time was three x 2 s in TCB to stop the incubation

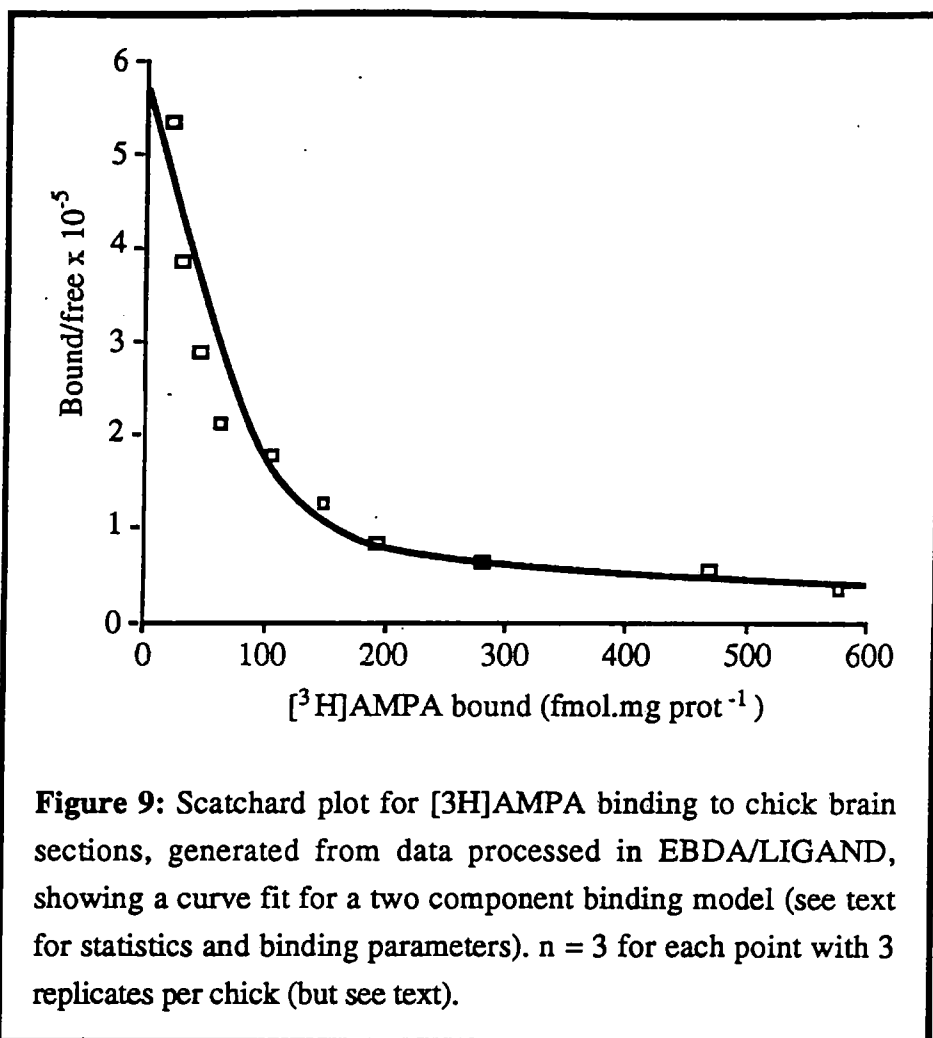
plus a dip wash in distilled water to remove buffer salts. Many of the sections incubated suffered significant damage, probably due to the high concentration of KSCN. At least one section from each triplicate, however, survived well enough to measure bound radioactivity using liquid scintillation counting (chapter 2 section 5.3.4 and experiment 1).

Results

The accurate maximum concentration of [^3H]AMPA used in this experiment (calculated as described in experiment 1) was found to be $194.35 \text{ nmol l}^{-1}$. s.a for the sample was 133.2 dpm/fmol . Data were processed in EBDA/LIGAND where Scatchard and Hill analyses were performed (see figure 9 for Scatchard plot). The NSB value, $28.6 \pm 6.3 \text{ dpm}$ was taken to represent zero specific binding and was subtracted from all other binding values during processing in EBDA/LIGAND. Scatchard and Hill analyses revealed two components of binding. Statistical analysis in LIGAND indicated that a two site model represented a significant improvement of fit compared with a one site model ($F_{7,5} = 7.76$, $p = 0.02$). Equilibrium binding parameters calculated in EBDA/LIGAND were: High affinity $K_D = 17.1 \pm 5.3 \text{ nmol l}^{-1}$, high affinity $B_{\text{max}} = 98.4 \pm 20.7 \text{ fmol.mg prot}^{-1}$; low affinity $K_D = 963 \pm 76 \text{ nmol l}^{-1}$, low affinity $B_{\text{max}} = 845 \pm 115 \text{ fmol.mg prot}^{-1}$, $nH = 0.59 \pm 0.06$.

Discussion

Unfortunately, many of the sections used in this experiment deteriorated physically during incubation. As a result of this most of the points on the Scatchard plot in figure 9 represent data from only a single brain. This deterioration was probably due to the high concentration of KSCN used, so in future experiments a much lower KSCN concentration should be used. The upshot of this extensive tissue deterioration is that the calculated binding parameters, at best can only be considered as an approximation. Nevertheless, these are reasonably similar to values found in much more thorough investigations using mammalian brain tissue⁸⁰, where two components of binding were also found. The range of concentrations used was apparently appropriate for Scatchard analysis and it was decided that a similar range could be used for further, autoradiographic experiments.



8

Preliminary quantitative autoradiography studies.

The data discussed in this section were generated by densitometric analysis of autoradiograms prepared by binding NMDA sensitive [^3H]L-glutamate, [^3H]glycine, [^3H]MK-801 or [^3H]AMPA to sections cut from anterior and intermediate forebrain locations of day old chicks (see chapter 1, figure 3), killed either 30 minutes or 3 hours after passive avoidance training. Autoradiograms of triplicate coronal sections from anterior and intermediate forebrain locations (see chapter 1 figure 3, b and c), for all four radioligands, were prepared from the brains of the same 18 chicks (9 MeA and 9 W) killed 30 min post-training, or the same 22 chicks (11 MeA and 11 W) killed 3 h post-training, using the techniques described in chapter 2, section 5.3 (see acknowledgements). Data from chicks killed 3 h post-training has been published in abstract form as Steele et al (1993)²⁰¹.

Table 3: Chick brain regions (see list of abbreviations and figure 3 in chapter 1) measured in the experiments described in sections 8.1 and 8.2. Regional radioligand binding density was measured by densitometry of autoradiograms prepared using coronal sections cut from the forebrains of day old chicks, killed either 30 min or 3 h after passive avoidance training.

anterior regions measured (30 min post- training autoradiograms)	intermediate regions measured (30 min post- training autoradiograms)	anterior regions measured (3 h post-training autoradiograms)	intermediate regions measured (3 h post-training autoradiograms)
LPO PA E MN LN MHV LHV HA	FPL AA PA Se E N TPO IMHV Hp HA	LPO PA E MN LN MHV LHV HD HIS HA	FPL AA PP PA Se N TPO IMHV Hp HA

8.1

Quantitative autoradiographic demonstration of changes in radioligand binding to NMDA but not AMPA receptors in day old chick forebrain 30 minutes after passive avoidance training.

Much of the data presented in this section has been published as Stewart et al (1992)²⁰⁵.

One of the stated aims of this research project was to further establish the role of L-glutamate receptors in memory for passive avoidance training (chapter 1). Part of the experiment described below has already been briefly discussed in chapter 1. Its aims were preliminary to that stated above in that it was designed to investigate whether any changes in binding of a number of radioligands to NMDA or AMPA receptors, can be detected during the early stages of memory formation after passive avoidance training and in which regions they occur.

8.1.1: Densitometric measurement of autoradiograms and data processing.

Densitometry of autoradiograms was performed using a Joyce-Loebl Magiscan MD image analysis system, as described in chapter 2 section 5.3.7. The binding densities of NMDA sensitive [^3H]L-glutamate, [^3H]glycine, [^3H]MK-801 and [^3H]AMPA, to a total of 18 forebrain regions were measured, in left and right hemispheres of each brain (8 from anterior sections and 10 from intermediate sections, see table 3 and figure 3, b and c) All 18 regions could not be measured for each of the four radioligands, due to variations in the extent and boundaries of optical density in some regions. Four triplicate sections (two brains from each group, MeA and W) from the NMDA sensitive [^3H]L-glutamate autoradiograms had to be disregarded due to poor quality. Background readings from the image analysis system were subtracted, triplicate means were taken, data was sorted by region, non-specific binding data was subtracted and data was standardized as described in chapter 2 section 5.3.7. All measurements and operations on data, until this point, were performed blind. Finally the data were sorted by code into MeA and water control groups. This gave two sets of nine values (W and MeA) for each region measured in each hemisphere. The means, standard deviations and standard errors of the mean for these were then calculated.

Analyses of regional binding differences between W and MeA chicks were performed using a two way analysis of variance (ANOVA) with a split plot design and t-tests, to find whether any of the differences were statistically significant. These statistical analyses were performed using specially prepared Microsoft Excel worksheets.

8.1.2: Results

The binding distributions of NMDA sensitive [^3H]L-glutamate, [^3H]MK-801 and [^3H]AMPA, observed in this study were very similar to those reported by Henley et al(1989)⁸⁴(see chapter 1, section 4.4). Examples are shown in figure 10. The highest levels of binding to NMDA receptors, of [^3H]L-glutamate and [^3H]MK-801, in the forebrain were in the neostriatum, hippocampus, hyperstriatum ventrale (HV) and archistriatum. Binding levels in the PA and HA were a little lower and binding in the HIS, HD and LPO was lower

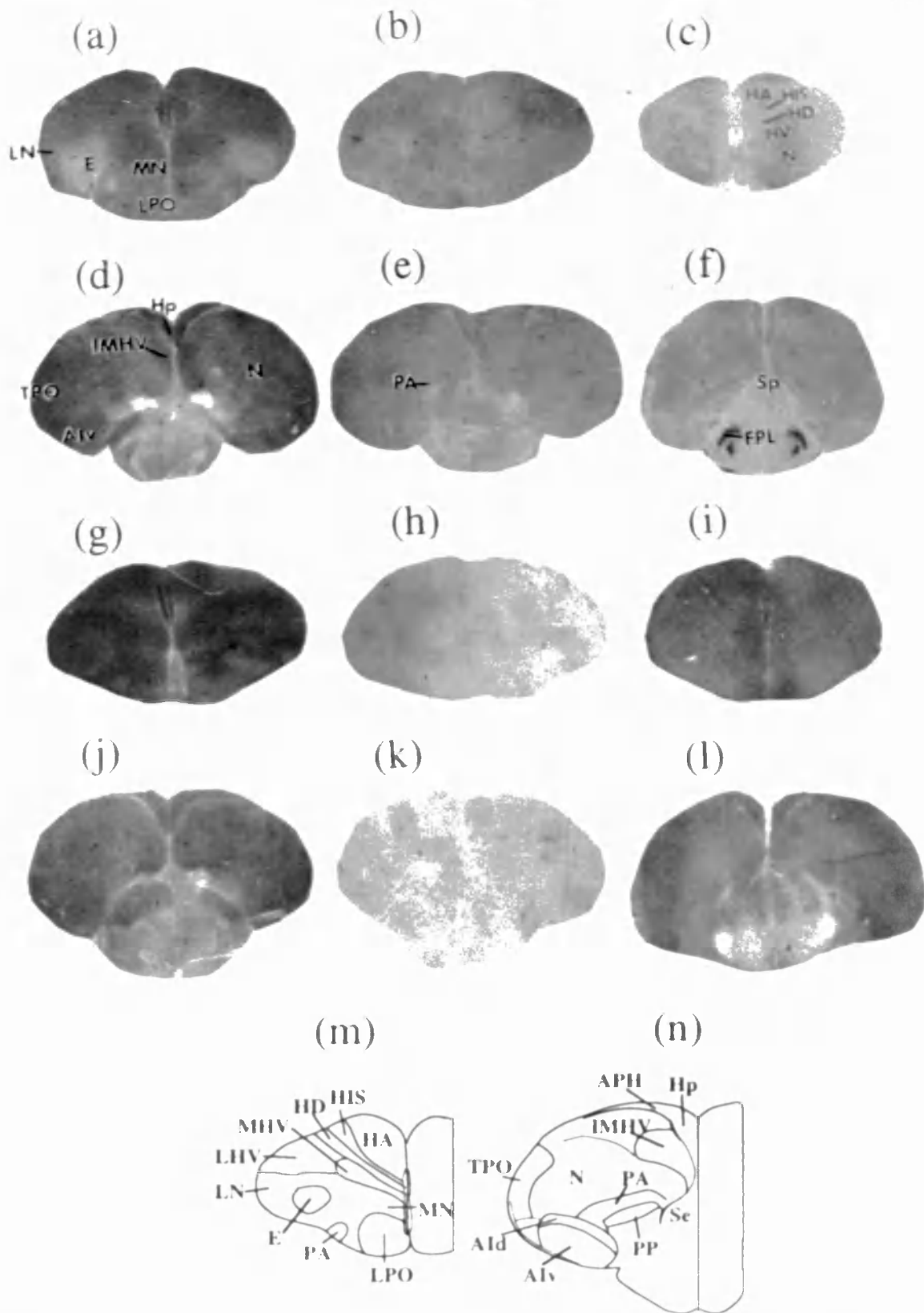


Figure 10: Photographs showing examples of autoradiograms from which measurements of regional radioligand binding were made. (a & d) Total [^3H]L-glutamate. (b & e) Non-specific binding (NSB) with NMDA as displacer. (c & f) [^3H]glycine. (g & j) Total [^3H]MK-801. (h & k) MK-801 NSB. (i & l) [^3H]AMPA, (m & n) chick forebrain regions. NSBs for [^3H]AMPA and [^3H]glycine were not visible above background.

still. Also binding in the LPO was not homogeneously distributed, tending to decrease in a lateral to medial gradient. The lowest level of binding in the forebrain was to be found in the ectostriatum, although the overall level of binding in the thalamus was much lower than this and was particularly low in some of the thalamic nuclei such as the fasciculus prosencephali lateralis (FPL). The binding distribution of [^3H]glycine in the forebrain was almost identical to that of NMDA sensitive [^3H]L-glutamate and of [^3H]MK-801. In the thalamus, however, binding levels were much higher and were, for the most part comparable to the forebrain levels, except in the FPL where the binding level was much higher than in any other thalamic or forebrain region. One possible explanation for this is that many thalamic neurons may contain inhibitory, strychnine sensitive, glycine receptors. It was decided that this merited further investigation (see section 8.3).

The distribution of [^3H]AMPA binding (figure 10, table 4) was also found to be very similar to that described by Henley et al (1989)⁸⁴, with the highest binding levels occurring in the hippocampus and archistriatum. The level of binding in other forebrain regions was a little lower, but fairly homogeneous. The lowest level of binding in the forebrain was, again, in the ectostriatum and the lowest binding level of all was again in the thalamus.

ANOVA indicated that significant differences in the binding of NMDA sensitive [^3H]L-glutamate, between MeA and W trained chicks, occurred in the IMHV ($F_{1,12} = 5.4$, $p = 0.039$), LPO ($F_{1,12} = 10.21$, $p = 0.008$), and in the lateral neostriatum (LN) ($F_{1,12} = 9.34$, $p = 0.01$). t-tests showed that these could be attributed to a significant increase in binding to the left LPO (34.3%; $t = 3.59$, $df = 12$, $p = 0.004$) and the left IMHV (38.6%; $t = 2.94$, $df = 12$, $p = 0.012$), and a significant decrease in the right LN (44%; $t = 6.74$, $df = 12$, $p = 0.00002$) (figure 11, a).

For [^3H]MK-801 binding, ANOVA indicates that significant differences, between MeA and W birds, occurred in the IMHV ($F_{1,16} = 19.04$, $p = 0.0005$), and that hemispheric interaction (ie, the binding change in one hemisphere, after training, compared to that in the other) was significant in the LPO ($F_{1,1} = 205.3$, $p = 0.044$). t-tests showed that these can be

Table 4: Data for binding of radioligands to coronal chick forebrain sections, 30 min after passive avoidance training (as described in section 8.1)

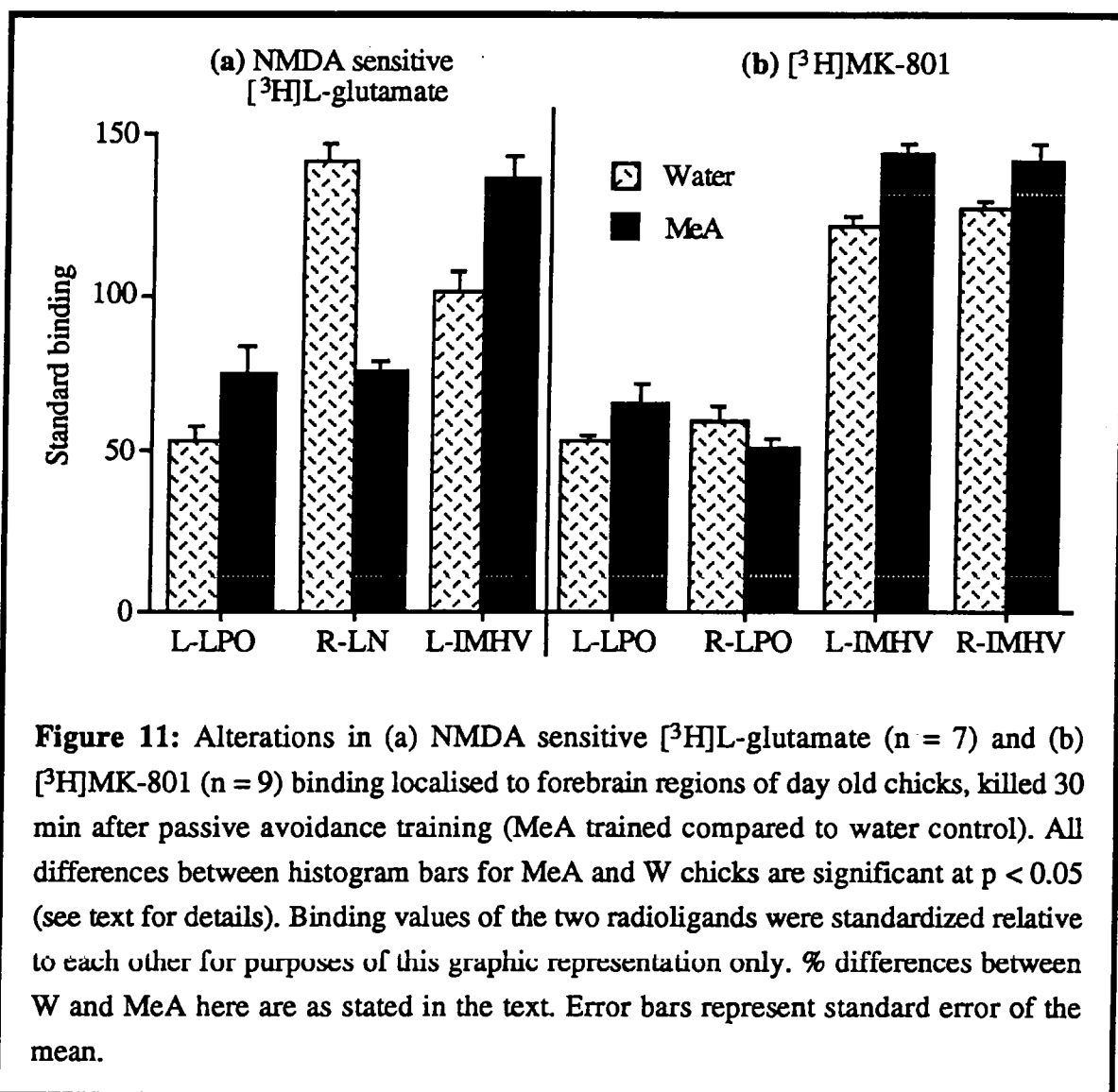
Binding Of:	NMDA sensitive [^3H]glutamate				[^3H]MK-801			
Condition	Control		Trained		Control		Trained	
Hemisphere	Left	Right	Left	Right	Left	Right	Left	Right
(a) Anterior brain regions								
LPO	113±0.9	143±0.7	152±6.0	140±0.9	25.7±1.0	28.9±7	31.5±9.0	25.1±1.4
PA	9.0±0.9	12.9±1.3	10.3±1.1	10.4±1.2	26.5±0.8	27.2±1.2	27.4±1.7	24.2±1.3
E	1.3±2.0	0.4±5.6	4.3±2.5	0.3±2.8				
MN					26.4±2.3	27.9±1.9	29.5±1.4	30.6±1.3
LN	23.7±2.1	29.8±0.9	21.0±2.3	20.7±0.9	62.4±2.5	60.7±1.4	61.3±3.1	59.3±2.9
MHV	21.6±2.3	24.9±1.1	21.3±1.0	22.0±0.8	48.1±1.5	52.1±0.9	47.2±2.4	49.8±2.5
LHV	17.7±1.7	22.3±0.9	18.5±1.0	19.9±0.8	56.2±2.3	58.5±2.1	55.3±1.1	54.5±0.8
HA	24.8±2.4	27.1±1.1	22.9±1.1	22.9±0.8	54.6±1.8	55.7±2.2	55.6±2.6	53.7±2.1
(b) Intermediate brain regions								
FPL	1.4±0.3	1.7±0.4	1.7±0.3	1.5±0.3	0.7±0.2	0.9±0.1	1.0±0.1	1.1±0.1
AA	3.5±1.7	10.2±2.1	9.0±3.0	6.1±1.5	48.7±3.8	52.5±4.0	35.9±4.7	42.1±5.3
PA	3.1±1.1	8.6±1.1	6.1±1.2	8.2±0.7	42.5±2.7	46.0±3.2	47.9±2.8	43.5±2.1
E	2.1±1.2	5.6±1.5	1.4±2.2	3.1±1.7				
Se					14.6±0.6	15.4±0.6	14.5±0.9	14.7±0.9
N	10.1±0.8	13.5±1.3	9.2±1.1	11.9±1.0				
TPO	9.4±1.2	15.1±1.1	15.1±2.8	12.7±1.5	61.4±2.6	66.6±2.3	68.3±2.4	60.2±2.3
IMHV	9.4±0.5	12.6±0.9	13.1±1.1	12.6±0.6	64.1±1.6	66.7±1.1	76.3±1.6	74.6±2.7
Hp	11.2±1.4	14.6±1.7	11.4±2.6	10.6±1.8	43.2±1.7	42.7±1.2	46.1±1.3	44.3±1.1
HA	12.8±0.6	17.1±0.4	13.4±1.1	14.9±1.5	53.2±3.1	59.5±3.5	51.8±3.3	54.4±3.5

Binding Of:	[^3H]glycine				[^3H]AMPA			
Condition	Control		Trained		Control		Trained	
Hemisphere	Left	Right	Left	Right	Left	Right	Left	Right
(a) Anterior brain regions								
LPO	5.0±0.2	4.6±0.2	4.7±0.2	4.4±0.1	44.6±1.3	43.2±2.6	41.7±3.0	39.0±2.6
PA	5.9±0.7	5.4±0.5	4.4±0.4	4.6±0.3	28.3±2.6	30.6±2.0	24.9±2.5	30.9±1.6
E	3.0±0.1	3.1±0.2	2.7±0.2	3.0±0.2				
MN	6.5±0.2	6.3±0.3	6.7±0.3	6.5±0.4	36.2±2.6	38.0±2.1	42.9±2.7	40.3±2.2
LN	5.8±0.2	4.9±0.2	5.5±0.2	5.4±0.3	37.5±2.4	43.2±2.3	38.7±2.1	42.1±1.6
MHV	7.3±0.6	6.6±0.4	7.4±0.3	7.2±0.4	64.5±2.5	68.0±4.4	80.3±9.2	70.1±2.9
LHV	5.1±0.1	4.3±0.3	4.5±0.2	5.1±0.2	61.1±2.9	66.5±2.4	58.7±4.4	75.9±4.2
HA	6.1±0.4	5.9±0.4	7.1±0.4	6.7±0.4	62.5±2.9	70.7±4.7	82.2±10.5	77.6±3.8
(b) Intermediate brain regions								
FPL	16.4±2.5	13.0±2.2	13.9±1.0	11.6±0.9	9.8±3.0	8.2±3.0	18.1±5.8	11.2±3.5
AA	2.6±0.1	2.4±0.1	2.5±0.1	2.6±0.1	61.3±9.8	61.0±5.7	42.5±4.6	52.6±7.3
PA	3.8±0.4	3.5±0.5	3.3±0.2	2.9±0.2	42.6±2.8	47.2±2.8	43.1±2.3	45.3±2.8
E								
Se	1.6±0.1	1.6±0.4	1.6±0.3	1.6±0.04	35.7±2.4	38.2±2.8	31.7±1.3	34.3±1.8
N	2.8±0.2	2.4±0.2	3.0±0.2	2.7±0.1				
TPO	2.6±0.2	2.5±0.2	2.5±0.1	2.7±0.1	46.4±2.8	62.1±4.2	47.7±2.7	52.2±3.3
IMHV	2.5±0.2	2.3±0.2	2.6±0.1	2.5±0.1	80.8±3.4	89.7±6.3	82.2±2.5	83.9±6.1
Hp	2.0±0.2	2.0±0.1	2.0±0.1	2.2±0.1	71.1±3.4	69.6±2.6	68.2±3.3	69.0±3.5
HA	2.7±0.2	2.5±0.3	2.5±0.1	2.7±0.2	64.7±2.8	71.8±4.2	67.5±1.5	64.6±2.4

Values were calculated in fmol.mg prot⁻¹ ± standard error of the mean. n = 9 for both water and MeA chicks. See figure 11 and text for significant differences between water and MeA conditions for NMDA sensitive [^3H]L-glutamate and [^3H]MK-801 binding. No differences between water and MeA for glycine or AMPA binding were found to be significant.

attributed to significant increases in binding to the left IMHV (19%; $t = 5.41$, $df = 16$, $p = 0.00006$), and right IMHV (11.8%; $t = 2.64$, $df = 16$, $p = 0.018$), as well as the left LPO (22%; $t = 3.59$, $df = 16$, $p = 0.003$), but a decrease in binding to the right LPO (15%; $t = 2.46$, $df = 16$, $p = 0.026$) (figure 11, b).

ANOVA, and t-tests, showed no significant differences in [^3H]glycine or [^3H]AMPA binding, between W and MeA chicks.



8.1.3: Discussion

It is clear from these results that passive avoidance training associated changes in binding to NMDA, but not AMPA receptors, can be detected using quantitative autoradiography 30 min post-training and that these changes are localized to distinct regions

of the chick brain. Specifically, increases in binding in the LPO and IMHV, in the left hemisphere particularly, and a decrease in binding to these receptors in the right LN, a region not previously implicated in chick memory and learning. As discussed in chapter 1 sections 4.3 and 4.4 the IMHV and the LPO both have been implicated in memory formation for passive avoidance training in many different studies. The results presented here indicate that up-regulation of NMDA receptors is involved in the early stages of memory formation for the passive avoidance task, but that AMPA receptors are apparently not.

Firm conclusions cannot be drawn from this study alone, but when considered in tandem with the findings of behavioural pharmacological studies such as that of Burchuladze and Rose (1991)³⁵, where NMDA receptor antagonists blocked memory formation but AMPA receptor antagonists did not, (see chapter 1, section 4.4 for details) the findings strongly suggest an important role for NMDA but not AMPA receptors in the early stages of learning in the chick. It is important however to distinguish between the two possible modes of up regulation of NMDA receptors, ie an increased number of receptors in a region or increased receptor affinity. A preliminary study of the equilibrium kinetics of NMDA and CPP sensitive [³H]L-glutamate binding 30 min after passive avoidance training (using Scatchard and Hill analyses) was, in fact, carried out. The data, however, are incomplete and are not presented here because insufficient data points were obtained to allow a precise estimate of binding parameters. Nevertheless, they do suggest that the binding alterations observed in this study are due to increased receptor numbers rather than altered affinity. It was always the intention to perform a full kinetic analysis of binding to NMDA receptors at 30 min post-training, but time constraints prevented this. Such analyses of NMDA receptor binding at 3 and 6.5 h post-training are, however, reported in chapter 5.

One seemingly anomolous result was that [³H]MK-801 binding increased bilaterally in the IMHV, whereas NMDA sensitive L-glutamate binding only increased in the left. In order to check whether this result was artifactual, the [³H]MK-801 binding assay was later repeated using brains from a different batch of chicks. The results were almost identical, showing significant increases in [³H]MK-801 binding bilaterally in the IMHV and in the left

LPO, the only difference being that no significant decrease in binding was observed in the right LPO. This strongly suggests that the observed increase in [^3H]MK-801 binding in the right IMHV is a genuine concomitant of passive avoidance training. It is possible that this difference between the observed alterations in NMDA sensitive [^3H]L-glutamate and [^3H]MK-801 binding may be an indication of the presence of two or more NMDA receptor subtypes in the chick forebrain (perhaps both bind MK-801, but one may be more sensitive to NMDA than the other). This possibility is considered further in chapter 5, and is further supported by evidence from the preliminary kinetic study mentioned above (data not shown), the results of which suggested increased CPP sensitive [^3H]L-glutamate binding in the right but not the left IMHV.

It is surprising that significant alterations in [^3H]glycine binding to NMDA receptors, similar to those found using NMDA sensitive [^3H]L-glutamate and [^3H]MK-801, were not observed. In fact, [^3H]glycine binding did increase in the left IMHV and the left LPO, but these increases were relatively small and were not significant in ANOVA or t-tests.

8.2

Quantitative autoradiographic demonstration of changes in radioligand binding to NMDA but not AMPA receptors in day old chick forebrain 3 hours after passive avoidance training.

The major aims of this experiment were to find whether the alterations in binding to NMDA receptors which were found to occur at 30 min after passive avoidance training would be repeated, or whether they were transient, to find whether alterations in binding to AMPA receptors could be found at this later time point, and to find whether any other regions which may be involved at a later stage in memory for passive avoidance training could be implicated.

8.2.1: Densitometric measurement of autoradiograms and data processing

This experiment was performed using techniques identical to those described in section 8.1.1, and the same four radioligands. In this case, however, 22 chicks were used (11 MeA trained and 11 water controls). The autoradiograms generated were of relatively high quality, which in all cases allowed measurement of binding from 20 regions of all 22 brains.

8.2.2: Results

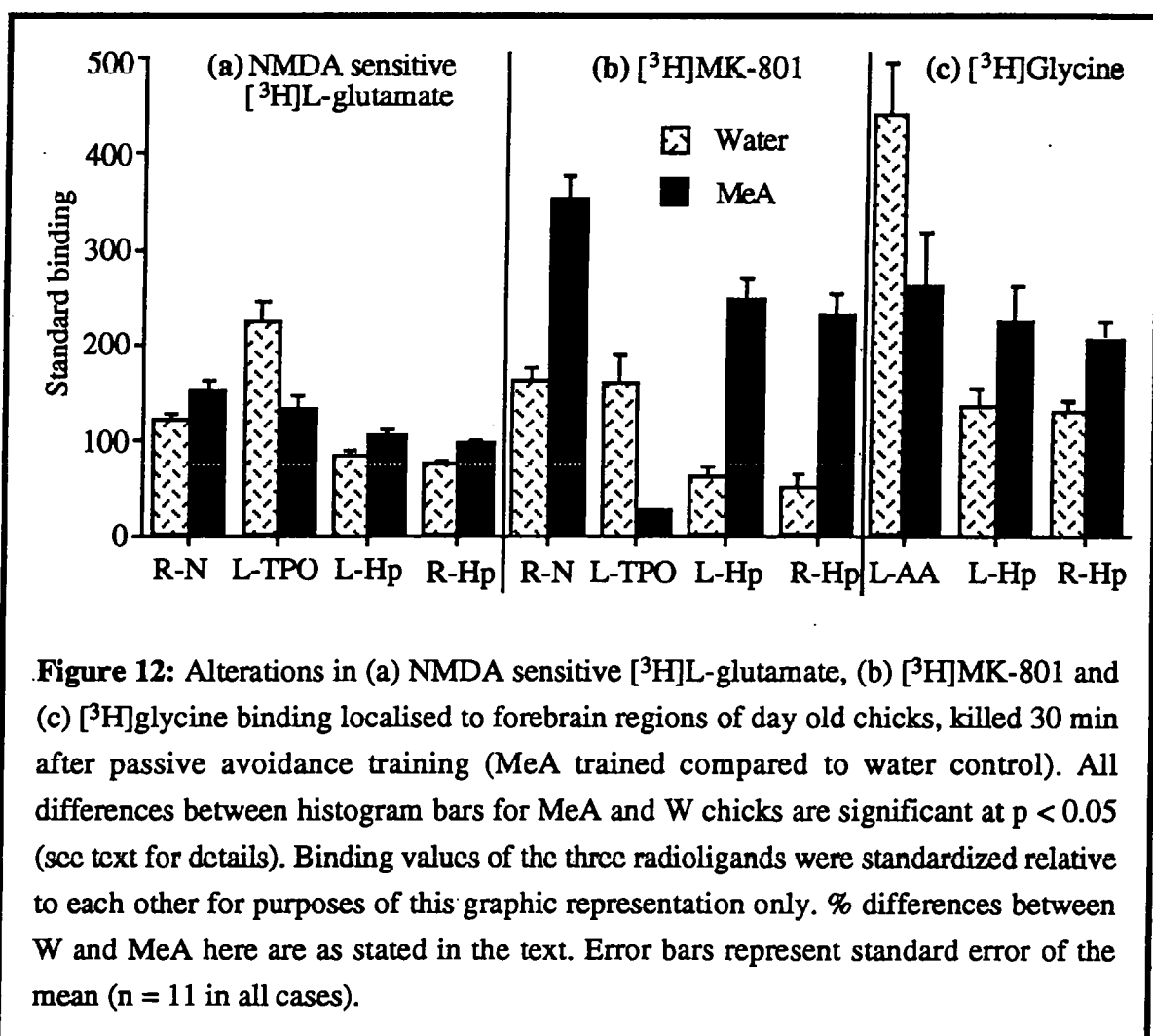
The binding distributions of all four radioligands were identical to those described in section 8.1.2.

ANOVA indicated that significant differences, between MeA and W chicks, in the binding of NMDA sensitive [^3H]L-glutamate, occurred in the neostriatum (N) ($F_{1,20} = 6.67$, $p = 0.018$), the TPO ($F_{1,20} = 4.74$, $p = 0.042$), and in the hippocampus (Hp) ($F_{1,20} = 5.62$, $p = 0.028$). t-tests indicated that these could be attributed to significant increases in binding to the right N (25%, $t = 2.62$, $df = 20$, $p = 0.016$), the left Hp (26.5%, $t = 2.09$, $df = 20$, $p = 0.0496$) and the right Hp (25%, $t = 2.41$, $df = 20$, $p = 0.026$) and to a significant decrease in the left TPO (69.7%, $t = 3.17$, $df = 20$, $p = 0.005$) (figure 12, a).

For [^3H]MK-801 binding, ANOVA indicated that significant differences, between MeA and W birds, occurred in the neostriatum ($F_{1,20} = 10.34$, $p = 0.004$), and the hippocampus, ($F_{1,20} = 62.3$, $p = 1.4 \times 10^{-7}$) and that a hemispheric interaction was significant in the TPO ($F_{1,1} = 11949$, $p = 0.006$). t-tests showed that these can be attributed to significant increases in binding to the right N (121%; $t = 6.7$, $df = 20$, $p = 1.6 \times 10^{-6}$), the left Hp (330%; $t = 8.2$, $df = 20$, $p = 8 \times 10^{-8}$) and the right Hp (367%; $t = 6.92$, $df = 20$, $p = 1 \times 10^{-6}$), but a decrease in binding to the left TPO (534%; $t = 4.17$, $df = 16$, $p = 0.0005$) accompanied by a smaller non-significant increase in the right (figure 12, b).

At this time point alterations in [^3H]glycine binding were also apparent. ANOVA indicated that that significant differences, between MeA and W birds, occurred in the Hp ($F_{1,20} = 7.65$, $p = 0.012$) and in the anterior archistriatum (AA) ($F_{1,20} = 4.5$, $p = 0.047$). t-tests showed that these can be attributed to significant increases, in binding, to the left Hp (69%, $t = 2.19$, $df = 20$, $p = 0.04$) and the right Hp (60%, $t = 3.55$, $df = 20$, $p = 0.002$), but a decrease in binding to the left AA (69%, $t = 2.28$, $df = 20$, $p = 0.034$) (figure 12, c).

There were no significant alterations in [^3H]AMPA binding.



8.2.3: Discussion

These results indicate that changes in binding to NMDA receptors (MeA trained compared to water controls) related to passive avoidance training, occurred only in localized

regions of the chick brain. At 3 hours after training, however, using both NMDA sensitive [^3H]L-glutamate and [^3H]MK-801, these changes were not only localized in different regions from those detected 30 minutes after training, but increases in binding were also lateralised, being more apparent in the right hemisphere than in the left. Apart from a bilateral increase in binding in the hippocampus and an increase in the right neostriatum, the only other alteration in binding found was a large decrease in the left TPO (figure 12). No significant differences were detected in any of the regions where changes occurred 30 minutes post-training.

Although they occurred in the same regions, the [^3H]MK-801 binding alterations, were disproportionately large compared to those found using NMDA sensitive [^3H]L-glutamate.

Significant alterations in [^3H]glycine binding were also observed 3 h post-training. These included a bilateral increase in binding in the hippocampus and a decrease in the left AA. The only region where changes in binding were consistently observed using all three of the NMDA receptor selective radioligands was the hippocampus. It is possible, therefore, that the alterations observed with NMDA sensitive [^3H]L-glutamate and [^3H]MK-801 in the right neostriatum and left TPO were statistically artifactual, but it can be stated with more confidence that the hippocampus is important for memory of the passive avoidance task at 3 h post-training, and that NMDA receptors have a key role in its memory related function. Also, the fact that [^3H]AMPA binding does not alter at either this or the 30 min time point indicates that AMPA receptors may not have a role in the early stages of memory formation or consolidation.

In summary, the results presented in sections 8.1 and 8.2 indicate a major temporal shift in the chick brain regions where NMDA receptors are involved in memory for the passive avoidance task, sometime during the period between 30 minutes and 3 hours post-training. Whereas the IMHV and LPO are important at 30 min post-training, the

hippocampus seems to be particularly important at the 3h time point. They also show that AMPA receptors are not involved up to, at least, 3 h post-training.

8.3

Inhibition of [³H]glycine binding in whole chick brain sections by glycine, 7-chlorokynurenate and strychnine

The possibility that strychnine sensitive [³H]glycine binding makes a significant contribution to the overall binding of [³H]glycine in the chick forebrain and/or thalamus, arose from the observed distribution of [³H]glycine binding to sections, discussed in section 8.1. The binding density, particularly in the thalamus, was observed to be quite different from that observed using other NMDA receptor selective radioligands. If strychnine sensitive binding accounts for a significant proportion of [³H]glycine binding in the forebrain it could act as a confounding factor, so that the [³H]glycine binding alterations found 3 h after passive avoidance training could not be attributed to up- or down-regulation of NMDA receptors with any confidence. The experiment described here was designed to test this by attempting to inhibit [³H]glycine binding to whole coronal chick brain sections containing either forebrain tissue only, or a high percentage of thalamic tissue, using strychnine as well as glycine and 7-chlorokynurenate (7-ClK) for comparison. 7-ClK is a highly selective antagonist of the glycine binding site on the NMDA receptor¹⁰¹.

8.3.1: Procedure

Forebrains from two untrained day old chicks were prepared as described in chapter 2 (section 5.3.1, see acknowledgements). Forty 10µm thick coronal sections of similar area, containing only forebrain tissue, and a further 40 containing a significant amount of thalamic tissue, were cut from each brain, thaw mounted on coverslips, dried and stored (chapter 2 section 5.3.2). This gave a total of 80 forebrain sections and 80 thalamic sections. Prior to pre-incubation, sections were allowed to come to room temperature. 75 forebrain sections (1 triplicate and 36 duplicates) and 75 thalamic sections were selected for incubation and

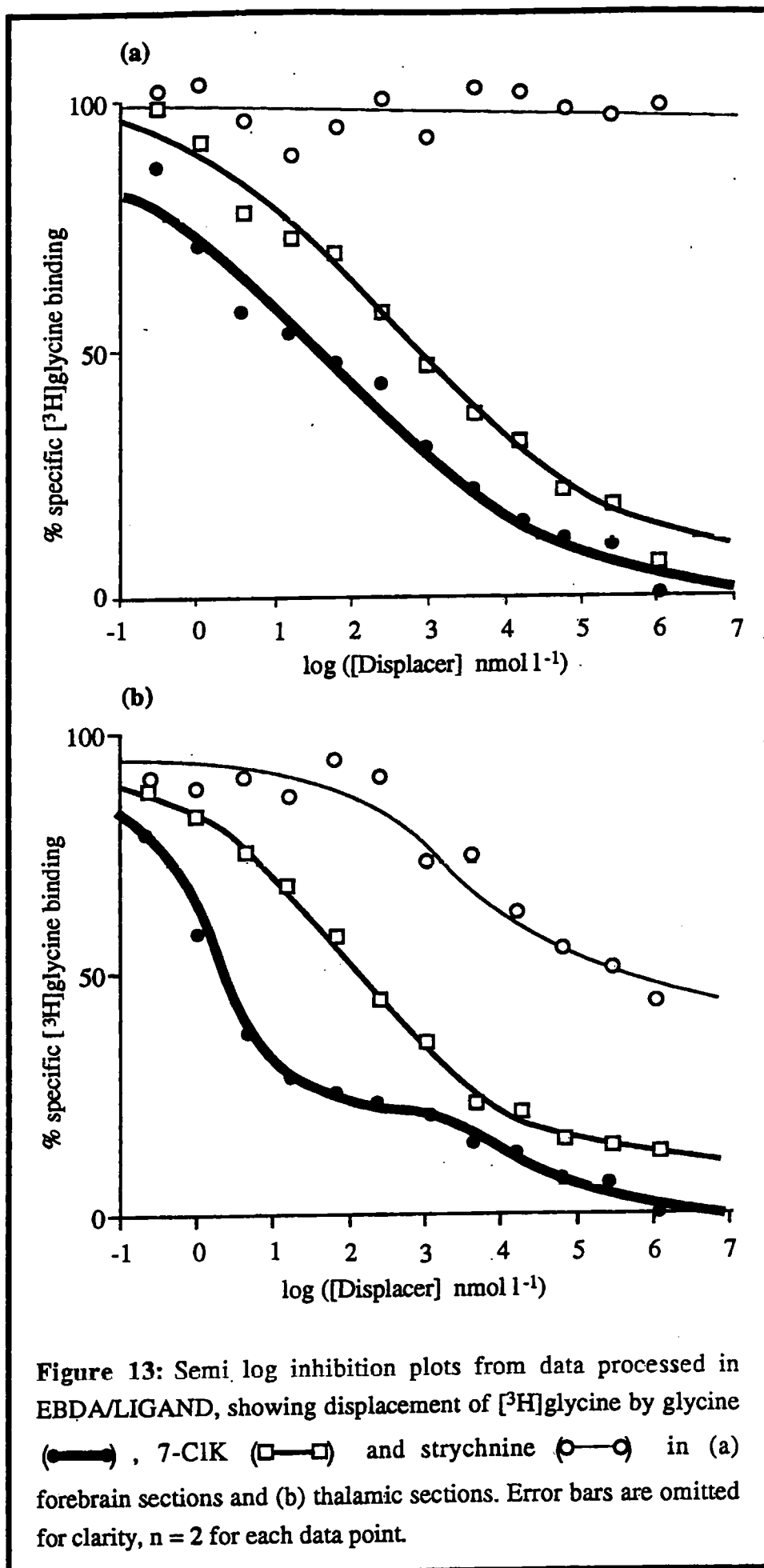
divided appropriately between 14 numbered racks. Sections were pre-incubated for one hour at 4°C in TCB, then dip washed in distilled water and dried in a cold air stream.

A range of 12 concentrations each of three displacers, glycine, 7-ClK and strychnine, were prepared (250 µl volumes) by 75% serial dilution (ie, 1 part displacer to 3 parts TCB) from a maximum concentration of 2 mmol l⁻¹. 250 µl of 200 nmol l⁻¹ [³H]glycine (in TCB) was then added to each displacer concentration. 350 µl of 200 nmol l⁻¹ [³H]glycine was also added to 350 µl of TCB for determination of total binding (To). This gave 37 solutions each containing 100 nmol l⁻¹ [³H]glycine, 36 of which contained serial dilutions of displacer (12 glycine, 12 7-ClK and 12 strychnine) ranging from 1 mmol l⁻¹ to 0.24 nmol l⁻¹, with the total binding solution acting as zero displacer for all three.

Sections were arranged appropriately on aluminium sheets (on ice) and incubations were performed as described in experiments 1 and 2 (sections 7.1.1 and 7.1.2). No significant damage to sections was observed and binding of [³H]glycine to sections and its accurate concentration in solution were measured using liquid scintillation counting as described in chapter 2, (section 5.3.4) and in experiment 1 (section 7.1.1).

8.3.2: Results

The accurate concentration of [³H]glycine used in this experiment was found to be 93.8 nmol l⁻¹, and s.a for the sample was 113.9 dpm/fmol. The activities of the forebrain triplicate and thalamic duplicate to which the highest concentration of glycine (1 mmol l⁻¹) was applied were considered to be NSB. These values were 146.4 dpm for forebrain and 152.2 for thalamic sections, and were taken to represent zero specific binding (100 % displacement) for all three displacers. They were subtracted from all other activity values during processing in EBDA to give specific binding values. Mean % specific binding was plotted against log[displacer] for each of glycine, 7-ClK and strychnine, and for both forebrain and thalamic sections (figure 13).



Inhibition of [^3H]glycine binding (compared to 100 % inhibition by 1 mmol l $^{-1}$ glycine) by 1 mmol l $^{-1}$ 7-ClK was $93.3 \pm 5.5\%$ in forebrain sections and $86.1 \pm 6.8\%$ in thalamic sections, whilst inhibition by strychnine was $1.6 \pm 6.3\%$ in forebrain sections and $51.4 \pm 6.1\%$ in thalamic sections. These values were calculated from binding data and not from the graphs shown in figure 13.

Statistical analysis in LIGAND indicated that a two component model represented a significant improvement of fit compared to a one component model for displacement by glycine in thalamic sections ($F = 2.72$, $p = 0.011$) although this was not true for forebrain sections, or for 7-ClK or strychnine in either type of section. IC $_{50}$ values generated by LIGAND in this case are not very meaningful as they are based on data from only two brains.

8.3.3: Discussion

Figure 13 (a) clearly shows that in forebrain sections strychnine displaces little or no [^3H]glycine, whilst the displacement curve for 7-ClK is similar to that for glycine. This suggests that glycine only binds to its site on NMDA receptors in day-old chick forebrain and that no inhibitory (strychnine sensitive) glycine receptors are present. In Figure 13(b) it can be seen that strychnine does inhibit [^3H]glycine binding to some extent in sections where there is a high proportion of thalamic tissue, and that this seems to be equivalent to the low affinity component of inhibition by glycine. This suggests that a relatively high proportion of [^3H]glycine binding in the thalamus is to inhibitory glycine receptors. This correlates well with the autoradiographic findings reported in section 8.1, where the distribution of [^3H]glycine binding in the forebrain was observed to be similar to that of NMDA receptors, but binding in certain thalamic nuclei (eg, the FPL) was very high, although only minimal numbers of NMDA receptors, if any, were shown to be present. It seems, therefore, that the passive avoidance training related alterations in [^3H]glycine binding in the forebrain, discussed in section 8.2, can be attributed to changes in the regional populations of NMDA receptors.

Chapter 4

The role of the NMDA receptor and its glycine co-agonist site in memory formation

9

NMDA receptors in memory for passive avoidance training: behavioural pharmacology studies.

Considerable evidence (chapter 1, sections 3.2 and 4.4) indicates that in both mammals and birds, the activation of NMDA receptors is a crucial initial step in memory formation^{35,141,226}. In chicks, evidence for the involvement of NMDA receptor activation in the early stages of memory formation, after training on the passive avoidance task, came from a behavioural pharmacology study by Burchuladze and Rose³⁵, with i.p or i.c injections of the use dependent, channel blocking, NMDA receptor antagonist MK-801 (section 2.1.2, section 7.1.3). I.c injections were made directly into the left or right IMHV. I.p injections either 1 h or 20 min pre-training had a significant amnesic effect when chicks were tested 3 or 24 h post-training, but no amnesia was apparent 30 min post-training. I.c injections into either the left or right IMHV, 5 min post-training, had a similar amnesic effect in birds tested 3 or 24 h post-training. Increased binding to NMDA receptors in the left IMHV, demonstrated in chicks tested 30 min post-training²⁰⁵ (section 8.1), however, suggests that the onset of amnesia induced by blocking NMDA receptors, might occur at an earlier time point than 3 h post-training. The present experiments were designed to investigate this possibility, and also the importance of the glycine co-agonist site on the NMDA receptor in memory formation, using the ligands 7-chlorokynurenate (7-CLK) or D-cycloserine (DCS).

It is possible that the drugs used could act at regions other than the IMHV. It seems likely, based on previous investigations of the diffusibility of drugs in the chick forebrain¹⁸¹, that a 5µl volume of MK-801, 7-ClK or DCS injected into the IMHV would diffuse to other regions in the dorsal part of the forebrain, aided by the fact that extracellular space is quite large in the brain of the newly hatched chick. The site of injection, therefore, cannot be taken to determine the sole region in which drugs act, although a high percentage of injected drug is likely remain near the injection site¹⁸¹.

9.1

The effect of i.c injections of MK-801 on memory for the passive avoidance task.

Introduction: in the study of Burchuladze and Rose³⁵ the published i.c injection concentrations were lower than the dissociation constants of the receptors for which they were intended (MK-801 concentration injected: 1.5 nmol l⁻¹, K_D of MK-801 binding site: 5 nmol l⁻¹ ⁹⁹). A further investigation of MK-801 as an amnesic drug in passive avoidance training, therefore, was made using a higher concentration. Also, ic injections were administered pre-training rather than 5 min post-training, because this is the post-training time point at which transient short term memory begins to be consolidated. A subconvulsive transcranial electroshock administered to a given number of chicks, 5 min post-training, results in approximately 50 % amnesia¹⁵⁷. Pre-training inhibition of NMDA receptors should, therefore, be more effective than 5 min post-training injections at inducing amnesia, assuming that NMDA receptors are, indeed, involved in the early stages of memory formation. All chicks in this experiment were injected i.c, directly into the IMHV as described in chapter 2 (section 5.2). Due to the thin unossified skull of day old chicks, these injections can be performed quickly and easily without any need for cutting of the skull. The chicks appear to suffer relatively little discomfort or distress and behave normally within seconds of being returned to their pens.

9.1.1: *Determination of dosage.*

Pairs of chicks were placed in pens, under the conditions described in chapter 2 (section 5.1). Chicks were pre-trained 3 times on a dry white bead, and then presented with a dry chrome bead to test pecking behaviour only. Those few chicks which did not show a natural tendency to peck were discarded. Chicks were then injected, bilaterally (5 μ l per hemisphere), with a range of MK-801 concentrations and were tested 30 min and 1 h later with a dry chrome bead. Chicks injected with MK-801 concentrations below 150 nmol l⁻¹ exhibited normal pecking behavior at white or chrome beads and had no obvious behavioural deficits. Of those chicks injected with 150 - 200 nmol l⁻¹ most failed to peck 30 min after injection, and all seemed rather drowsy and slightly uncoordinated. 1 h after injection, however, most seemed normal. Chicks injected with concentrations over 200 nmol l⁻¹ showed increasing behavioural deficits 30 min after injection and these were still apparent at 1 h. All were very uncoordinated and had little interest in pecking the bead. 30 min after 1 μ mol l⁻¹ injections, chicks were very lethargic and exhibited poor motor control. At 1 h most had recovered to some extent, but were still uncoordinated. 100 nmol l⁻¹ was, therefore, considered to be an appropriate concentration and was used in subsequent experiments.

9.1.2: *Training and injection schedule*

A series of three experiments in which injections were made bilaterally, or into the left or right IMHV only, were performed. Each consisted of two trials from which results were pooled. In each trial chicks were pre-trained, as described in section 9.1.1, and injected (i.c) with 5 μ l per hemisphere of either 100 nmol l⁻¹ MK-801 (in 0.9% sterile saline) or 0.9% sterile saline, 10 min after the third pre-training trial and 30 min prior to training on a MeA coated chrome bead. Chicks were tested using a dry chrome bead 1 h post-training, by a researcher who was unaware of the experimental history of the chicks. Those chicks which did not show a natural tendency to peck on pre-training, or failed to peck the training bead were discarded. In experiment 1 a total of 61 chicks were injected in

both the left and right IMHV (Trial 1: 14 MK-801, 14 saline, Trial 2: 17 MK-801, 16 saline). In experiment 2 a total of 40 chicks were injected in the left hemisphere only (trials 1 and 2: 10 MK-801, 10 saline). In experiment 3 a total of 40 chicks were injected in the right hemisphere only (trials 1 and 2: 10 MK-801, 10 saline). On testing chicks were scored as peck (amnesia) or avoid (recall). Results were analysed using $2 \times 2 \chi^2$ tests with a null hypothesis that MK-801 has no effect and, therefore, the number of MK-801 and saline injected chicks which avoid the test bead should be equal. $p < 0.05$ where $\chi^2 > 3.84$.

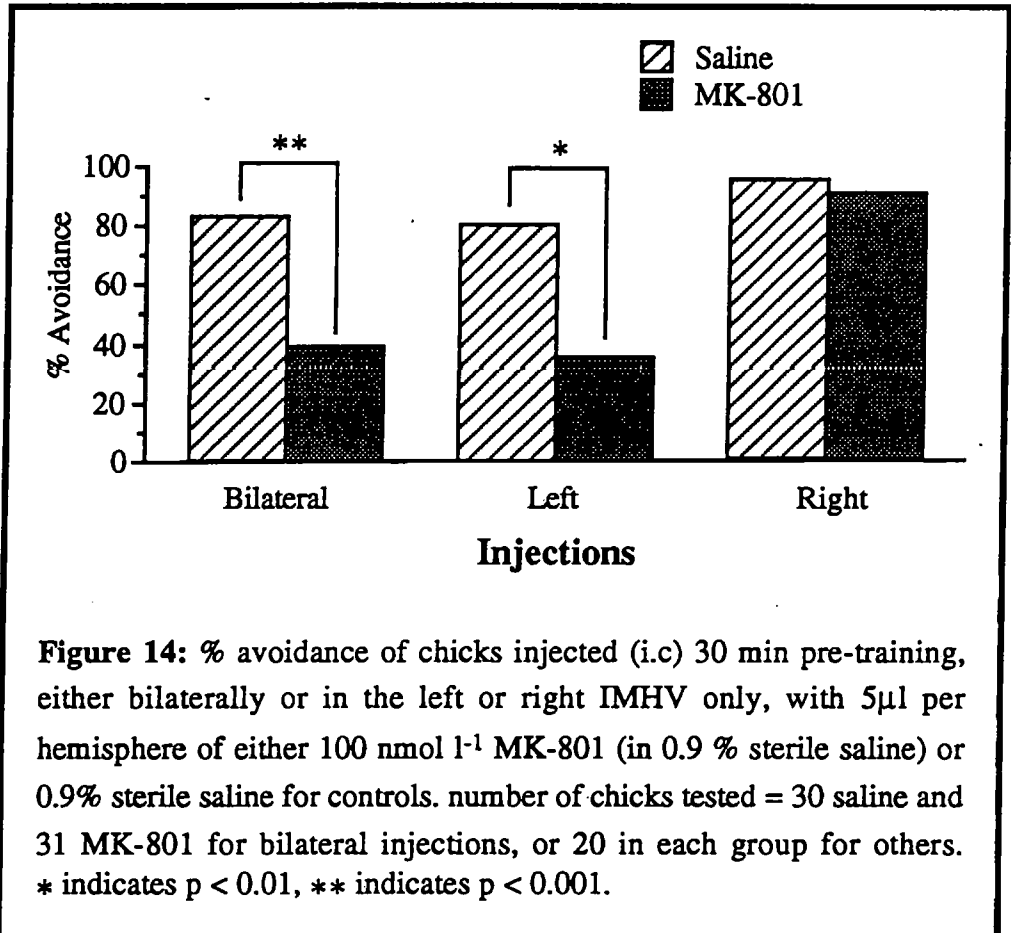
9.1.3: Results

These data are presented as histograms in figure 14. In experiment 1 (bilateral injections), 25 out of 30 saline injected chicks avoided (83 %) and 12 out of 31 MK-801 chicks avoided (39 %), $\chi^2 = 12.72$, $p < 0.001$. In experiment 2 (left IMHV injections), 16 out of 20 saline injected chicks avoided (80 % avoidance) and 7 out of 20 MK-801 chicks avoided (35 %), $\chi^2 = 8.82$, $p < 0.01$. In experiment 3 (right IMHV injections), 1 out of 20 saline injected chicks avoided (95 % avoidance) and 2 out of 20 MK-801 chicks avoided (90 %), $\chi^2 = 0.36$.

9.1.4: Discussion

These results show that MK-801 has a significant amnesic effect on chicks tested 1 h post-training, when injected bilaterally or in the left IMHV pre-training. It has no effect at this time point, however, when injected into the right IMHV pre-training. This means that pre-training blockade of NMDA receptors in the left, but not the right, IMHV results in amnesia for the passive avoidance task 1 h post-training, in contrast with the findings of Burchuladze and Rose³⁵ where amnesia was shown to result from either left or right injections, but not earlier than 3 h post-training. Further experiments performed in our laboratories (C. Hölscher, unpublished data) support both the results presented here and those, at the 3 h time point, of Burchuladze and Rose³⁵. Hölscher's results show that pre-training i.c injections of 100 nmol l^{-1} MK-801 were amnesic 30 min post-training, when

injections were made in the left IMHV but not in the right, but that identical pre-training injections resulted in amnesia 3 h post-training when injections were made in either hemisphere.



The data presented here, in combination with that of Hölscher and that of Burchuladze and Rose³⁵, confirms the involvement of NMDA receptors in memory formation for the passive avoidance task in chicks, and indicates that NMDA receptors are crucial for memory formation in the left IMHV as early as 30 min post-training, and certainly at 1 h post-training. The right IMHV either is not involved in memory formation at these time points, or utilizes processes which do not require NMDA receptors. The involvement of NMDA receptors in both the left and right IMHV, however, is necessary at 3 h post-training. This would indicate that memory related, NMDA receptor mediated, processes, begin in the right IMHV between 1 and 3 h post-training, somewhat later than in the left .

9.2

The effect of inhibiting the NMDA receptor's glycine site on memory formation, after passive avoidance training.

Data presented in this section were published as Steele and Stewart (1993)²⁰⁰

Introduction: NMDA receptor activation not only depends upon binding of the neurotransmitter L-glutamate and dissociation of Mg^{2+} from its channel, but also on binding of glycine to its allosteric co-agonist site^{117,124}. The involvement of NMDA receptor activation, early in memory formation for the passive avoidance task, as indicated by the data presented in section 9.1, together with the results of studies in mammals which indicate that the glycine co-agonist site of the NMDA receptor is essential for both LTP induction and memory formation^{67,153,193,210}, raised the strong possibility that the allosteric glycine site is also likely to have an important role in the induction of memory formation in the chick. In the following experiments, therefore, the effects of a glycine site antagonist, 7-ClK, on passive avoidance training was investigated. 7-ClK is a highly selective antagonist at the glycine site on the NMDA receptor^{101,103}. In mammals 7-ClK has been shown to inhibit hippocampal LTP¹⁵³ and to antagonize improvements in the rate of acquisition of hippocampal dependant classical conditioning, induced by treatment with glycine site partial agonists²¹⁰. Chicks were reared, pre-trained and trained using the conditions and procedures described in section 5.1 whilst injections were as described in section 5.2.

9.2.1: Determination of dosage

Pairs of chicks were placed in pens, pre-trained and presented with a chrome bead to test pecking behaviour as in section 9.1.1. Bilateral injections (5 μ l per hemisphere), with a range of 7-ClK concentrations (0.1 - 500 μ mol.l⁻¹), were administered and chicks were re-tested 30 min and 1 h later with a dry chrome bead. Chicks injected with 7-ClK concentrations below 200 μ mol l⁻¹ exhibited normal pecking behavior on either white or chrome beads and had no other behavioural deficits. Chicks injected with concentrations in

the range of 200 - 500 $\mu\text{mol l}^{-1}$ showed concentration dependent behavioural deficits 30 min after injection (mostly lack of coordination and drowsiness). At 1 h all chicks had recovered and no behavioural deficits were observed. 100 $\mu\text{mol.l}^{-1}$ 7-ClK was considered to be a satisfactory concentration (half that at which behavioural deficits became apparent) and was used in subsequent experiments, with 5 μl of 0.9% sterile saline for control injections.

9.2.2: Training and injection schedule

In a series of eleven experiments, groups of 30 chicks were placed in pens in pairs, (under conditions described in section 5.1) and pre-trained as described in section 9.1.1. Chicks which did not exhibit a natural tendency to peck were discarded. In experiments 1 - 9, half of the remaining chicks were injected with 7-ClK and half with saline, 30 min prior to training on an MeA coated bead. In experiments 10 and 11 injections were administered 5 min post-training. Chicks which failed to peck the training bead were discarded. In experiments 1, 4, 7, 10 and 11 injections were given in both hemispheres, in 2, 5 and 8 they were given in the left hemisphere only and in 3, 6 and 9, they were given in the right hemisphere only. Chicks were tested by presenting a dry chrome bead 30 min post-training in experiments 1, 2, 3 and 10; after 1 h in experiments 4, 5, 6 and 11, and after 3 h in experiments 7 - 9 (see table 5).

Results were analysed using 2×2 χ^2 tests, with a null hypothesis that 7-ClK has no effect and, therefore, the number of saline and 7-ClK injected chicks which avoid the test bead should be equal, $p < 0.05$ where $\chi^2 > 3.84$.

9.2.3: Results

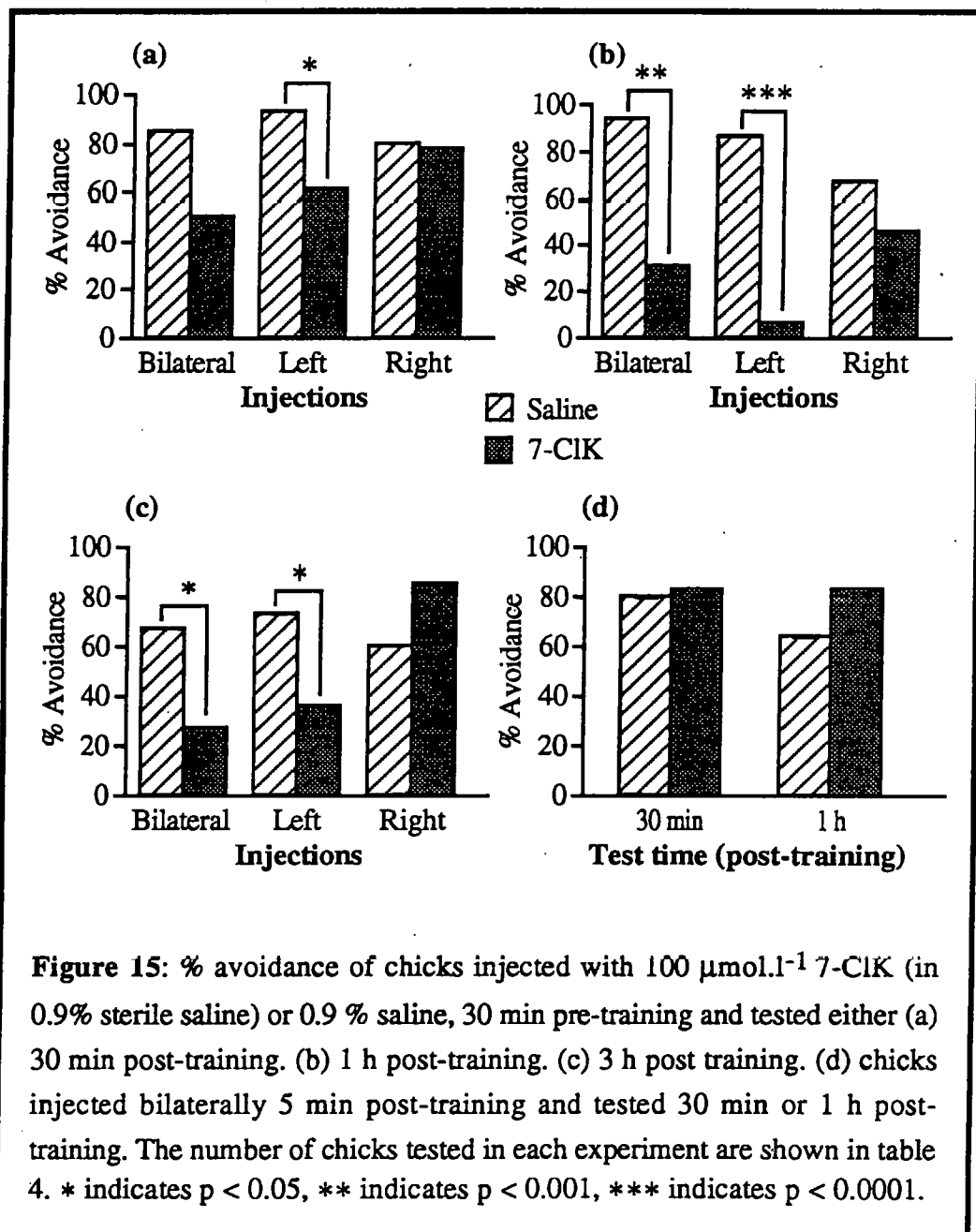
Pre-training injections: The data for experiments 1 - 3 are presented in figure 15 (a). In experiment 1 (bilateral injections, chicks tested 30 min post-training) 85 % of saline injected chicks avoided and 50 % of 7-ClK chicks avoided, $\chi^2 = 3.63$. In experiment 2 (left IMHV injections, chicks tested 30 min post-training) 93 % of saline injected chicks avoided

Table 5: Experimental design. Experiments 1-9: Chicks injected 30 min pre-training. Experiments 10 and 11: Chicks injected 5 min post-training. In all experiments half of the chicks were injected with 5 μ l of 100 μ mol l⁻¹ 7-ClK (in 0.9% sterile saline) per hemisphere and half were injected with 5 μ l of 0.9% sterile saline.

<u>Experiment number and figure reference</u>	<u>Hemisphere injected</u>	<u>Test time (post training)</u>	<u>Number of chicks tested</u>
1 (fig 15, a)	Both	30 min	saline: 13, 7-ClK: 14
2 (fig 15, a)	Left	30 min	saline: 15, 7-ClK: 13
3 (fig 15, a)	Right	30 min	saline: 10, 7-ClK: 9
4 (fig 15, b)	Both	1 h	saline: 15, 7-ClK: 13
5 (fig 15, b)	Left	1 h	saline: 14, 7-ClK: 15
6 (fig 15, b)	Right	1 h	saline: 15, 7-ClK: 13
7 (fig 15, c)	Both	3 h	saline: 12, 7-ClK: 15
8 (fig 15, c)	Left	3 h	saline: 15, 7-ClK: 14
9 (fig 15, c)	Right	3 h	saline: 15, 7-ClK: 13
10 (fig 15, d)	Both	30 min	saline: 10, 7-ClK: 12
11 (fig 15, d)	Both	1 h	saline: 11, 7-ClK: 12

and 61.5 % of 7-ClK chicks avoided, $\chi^2 = 4.18$, $p < 0.05$. In experiment 3 (right IMHV injections chicks tested 30 min post-training) 80 % of saline injected chicks avoided and 78 % of 7-ClK chicks avoided, $\chi^2 = 0.01$. The data for experiments 4 - 6 are presented in figure 15 (b). In experiment 4 (bilateral injections, chicks tested 1 h post-training) 93 % of saline injected chicks avoided and 31 % of 7-ClK chicks avoided, $\chi^2 = 11.87$, $p < 0.001$. In experiment 5 (left IMHV injections, chicks tested 1 h post-training) 86 % of saline injected chicks avoided and 7 % of 7-ClK chicks avoided, $\chi^2 = 18.29$, $p < 0.0001$. In experiment 6 (right IMHV injections chicks tested 1 h post-training) 67 % of saline injected chicks avoided and 46 % of 7-ClK chicks avoided, $\chi^2 = 1.2$. The data for experiments 7 - 9 are presented in figure 15 (c). In experiment 7 (bilateral injections, chicks tested 3 h post-training) 67 % of saline injected chicks avoided and 27 % of 7-ClK chicks avoided, $\chi^2 = 4.32$, $p < 0.05$. In experiment 8 (left IMHV injections, chicks tested 3 h post-training) 73 % of saline injected chicks avoided and 36 % of 7-ClK chicks avoided, $\chi^2 = 4.14$, $p < 0.05$. In experiment 9 (right IMHV injections chicks tested 3 h post-training) 60 % of saline injected chicks avoided and 85 % of 7-ClK chicks avoided, $\chi^2 = 2.01$ (figure 15, c).

Five min post-training injections (figure 15, d): In experiment 10 (bilateral injections, chicks tested 30 min post-training), 80 % of saline injected chicks avoided and 83 % of 7-ClK chicks avoided, $\chi^2 = 0.63$. In experiment 11 (bilateral injections, chicks tested 1 h post-training) 63.6 % of saline injected chicks avoided and 83 % of 7-ClK chicks avoided, $\chi^2 = 2.65$ (figure 15, d).



9.2.4: Discussion

These data indicate that injection of 7-ClK into the left but not the right IMHV, prior to training, has a significant amnesic effect on chicks trained on the passive avoidance task. The finding that 7-ClK blocks memory formation in the left, but not the right hemisphere is in accordance with previous data, which suggests that the left IMHV plays a crucial role in the initial phases of memory formation for the avoidance response^{177,202,205}. The data of Burchuladze and Rose³⁵ and Hölscher (unpublished data) discussed in section 9.1, however, has shown that right IMHV injections of MK-801 result in amnesia 3 h post-training. A possible explanation for this is that different NMDA receptor subtypes may be involved in memory formation in the left and right IMHV, with receptors in the left IMHV more sensitive to 7-ClK than in the right, whereas MK-801 may be equally effective in blocking both subtypes. This possibility will be investigated further in chapter 5, but is supported by the autoradiographic data presented in chapter 3 (section 8.1)²⁰⁵, which demonstrates that NMDA sensitive [³H]L-glutamate binding increases only in the left IMHV after passive avoidance training, whereas [³H]MK-801 binding increases in both left and right. Also, in contrast to MK-801³⁵ (section 9.1), 7-ClK does not result in amnesia when injected 5 min post-training in either left or right IMHV. One possible explanation for this, involves the probability that inhibition of the glycine site on the NMDA receptor by 7-ClK does not completely inhibit the function of the receptors, so that post-synaptic Ca²⁺ entry is reduced rather than blocked. When pre-training injections are administered, therefore, such a reduction in Ca²⁺ influx would be enough to block initiation of the biochemical processes associated with memory formation, but when injections are given 5 min post-training, a proportion of NMDA receptors may already have been activated and the reduced Ca²⁺ influx would not be sufficient to prevent the Ca²⁺ concentration from reaching a threshold where memory related processes would be initiated. These results indicate nevertheless, that in the chick, as in mammalian species, the glycine co-agonist site on the NMDA receptor plays an important role in the early stages of memory formation.

9.3

The effect of D-cycloserine, a partial agonist of the glycine site NMDA receptor, on memory formation after passive avoidance training on a weakly aversive stimulus.

Introduction: the data presented above indicate that activation of the glycine site on the NMDA receptor, in the left IMHV, may be necessary for memory formation after passive avoidance training. This was further investigated using a drug which acts as a partial agonist at this site, D-cycloserine (DCS)⁹³. DCS has been shown to improve the rate of acquisition of a hippocampus-dependant classical conditioning task in rabbits²¹⁰, and also to reduce learning and memory deficits induced by antagonists of the cholinergic system in rats^{67,193}.

After passive avoidance training, where MeA is used as the aversive stimulus, approximately 85% of chicks avoid the bead on re-testing after 30 min, and this continues up to 24 h post training¹⁸¹, showing that short term memory for the task has a high probability of being consolidated into long term memory. Where a weaker stimulus is used, such as quinine²⁸ or a dilution of MeA¹⁸¹, memory for the passive avoidance task has a much lower probability of being consolidated, and significantly fewer chicks subsequently remember to avoid the test bead^{28,181}, eg, Rosenzweig et al¹⁸¹ have shown that less than 50 % of chicks trained on 10 % MeA show recall at 30 min post-training, compared to 85 % with 100 % MeA. Administration of a ligand, such as DCS, which helps to activate NMDA receptors, at the time point when memory consolidation begins to occur, ie, within 5 min post-training, may make memory consolidation more probable where a weak aversive stimulus is used. Although DCS is a partial agonist which does not fully activate the glycine co-agonist site on NMDA receptors, a relatively high concentration is likely to potentiate the activation of a high enough proportion of NMDA receptors to influence learning, whilst avoiding any of the neurotoxic effects which may result from the use of a full agonist.

Chicks were reared, pre-trained and trained using the conditions and procedures described in section 5.1, but the training bead was coated with a weak aversive stimulus rather than MeA. An initial series of experiments was performed using a saturated solution of quinine in 20 % ethanol, but experiments involving post-training injections were later repeated using 10 % MeA in ethanol. Injection procedures were as described in section 5.2.

9.3.1: Determination of dosage

Pairs of chicks were placed in pens, pre-trained and presented with a chrome bead to test pecking behaviour as in section 9.1.1. Bilateral injections (5 μ l per hemisphere), with a range of DCS concentrations (0.1 μ mol.l⁻¹ - 2 mmol l⁻¹), were administered and chicks were tested 30 min and 1 h later with a dry chrome bead. Chicks injected with DCS concentrations below 1.2 mmol l⁻¹ exhibited normal pecking behavior and had no other behavioural deficits. Chicks injected with concentrations in the range of 1.2 - 2 mmol l⁻¹ had behavioural deficits (drowsiness, lack of interest in the bead), the severity of which increased with concentration 30 min after injection, but less so at 1 h. 600 μ mol.l⁻¹ DCS (half of the lowest concentration which resulted in behavioural deficits) was considered to be satisfactory and was used in subsequent experiments, with 0.9% sterile saline for control injections.

9.3.2: Training and injection schedule

A series of experiments, each of which consisted of two trials from which results were pooled, were performed in which chicks were trained using a saturated quinine solution as a weak aversive stimulus. In each trial groups of chicks were placed in pens (under conditions described in section 5.1), two chicks in each, and pre-trained as described in section 9.1.1. Chicks which did not exhibit the natural tendency to peck were discarded. Bilateral ic injections of DCS or saline were administered to the remaining chicks, either 10 min prior to training, or 5 min post-training. Where bilateral injections resulted in a significant difference in avoidance between saline and DCS injected chicks, additional

experiments were performed in which chicks were injected in either the left or right IMHV only. Chicks were tested 1, 6 or 24 h post-training by presenting a dry chrome bead. The numbers of chicks tested were approximately 50 in most experiments (table 6 for details).

Table 6: Experimental design. Experiments 1-8: In all experiments approximately half of the chicks were injected, (i.c) directly into the IMHV, with 5 μ l of 600 μ mol.l⁻¹ DCS (in 0.9% sterile saline) per hemisphere and half were injected (i.c) with 5 μ l of 0.9% sterile saline per hemisphere.

<u>Figure where data are presented</u>	<u>Injection time (relative to training)</u>	<u>Weak aversant used</u>	<u>Hemisphere injected</u>	<u>Test time (post training)</u>	<u>Number of chicks tested</u>
16 (a)	10 min pre	Quinine	Both	1 h	24 saline 21 DCS
16 (a)	10 min pre	Quinine	Both	6 h	25 saline 23 DCS
16 (a)	10 min pre	Quinine	Both	24 h	13 saline 13 DCS
16 (b)	5 min post	Quinine	Both	1 h	25 saline 25 DCS
16 (c)	5 min post	Quinine	Both	6 h	25 saline 24 DCS
16 (c)	5 min post	Quinine	Left	6 h	33 saline 37 DCS
16 (c)	5 min post	Quinine	Right	6 h	34 saline 37 DCS
16 (b)	5 min post	Quinine	Both	24 h	23 saline 22 DCS
17 (a)	5 min post	10 % MeA	Both	1 h	28 saline 29 DCS
17 (a)	5 min post	10 % MeA	Left	1 h	24 saline 26 DCS
17 (a)	5 min post	10 % MeA	Right	1 h	26 saline 25 DCS
17 (b)	5 min post	10 % MeA	Both	6 h	24 saline 25 DCS
17 (b)	5 min post	10 % MeA	Left	6 h	20 saline 20 DCS
17 (b)	5 min post	10 % MeA	Right	6 h	20 saline 20 DCS
17 (c)	5 min post	10 % MeA	Both	24 h	24 saline 24 DCS

In a second series of experiments, each of which consisted of two trials from which results were pooled, chicks were trained using 10 % MeA as a weak aversive stimulus. Other experimental details were as above, except that in all trials chicks were injected 5 min post-training. See table 6 for details and schedule.

Results were analysed using 2×2 χ^2 tests, with a null hypothesis that DCS has no effect on memory retention and, therefore, the number of saline and DCS injected chicks which avoid the test bead should be equal, $p < 0.05$ where $\chi^2 > 3.84$.

9.3.3: Results

Quinine as weak aversant (figure 16 (a) - (c): when bilateral injections were given pre-training and chicks were tested 1 h, 6 h or 24 h post-training (figure 16, a) no significant increases in avoidance occurred (1 h: saline - 37.5 % avoidance, DCS - 47.6 % avoidance, $\chi^2 = 0.47$; 6 h: saline - 44 % avoidance, DCS - 30.4 % avoidance, $\chi^2 = 0.94$; 24 h: saline - 30.8 % avoidance, DCS - 53.9 % avoidance, $\chi^2 = 1.42$). This was also the case when post-training bilateral injections were given and chicks were tested 1 h or 24 h post-training (figure 16, (b) - 1 h: Saline - 68 % avoidance, DCS - 44 % avoidance, $\chi^2 = 2.92$; 24 h: saline - 43.5 % avoidance, DCS - 50 % avoidance, $\chi^2 = 0.19$). When post-training bilateral injections were given and chicks were tested 6 h post-training, however, (figure 16, c) 32 % of saline injected chicks avoided and 62.5 % of DCS chicks avoided, $\chi^2 = 4.57$, $p < 0.05$. Post-training, left IMHV injections of chicks which were subsequently tested at the 6 h time point also resulted in a significant increase in avoidance, whereas similar right IMHV injections did not (figure 16, (c) - left: Saline - 45.5 % avoidance, DCS - 70.3 %, $\chi^2 = 4.43$, $p < 0.05$; Right: saline - 44.1 % avoidance, DCS - 37.8 % avoidance, $\chi^2 = 0.29$).

10 % MeA as weak aversant (all chicks injected 5 min post-training): when chicks were injected bilaterally and tested 1 h post-training (figure 17, a), 28.6 % of saline injected chicks avoided and 55.2 % of DCS chicks avoided, $\chi^2 = 4.14$, $p < 0.05$. When tested at the same time point after injections in the left IMHV only, 29.2 % of saline injected chicks avoided and 57.7 % of DCS chicks avoided, $\chi^2 = 4.12$, $p < 0.05$, but similar right IMHV injections did not result in a significant increase in avoidance (saline: 42.3 % avoidance, DCS: 48 % avoidance, $\chi^2 = 0.17$ (figure 17, a). When chicks were injected bilaterally and tested 6 h post-training (figure 17, b), 41.7 % of saline injected chicks avoided and 72 % of DCS chicks avoided, $\chi^2 = 4.6$, $p < 0.05$. Again, when injections were given in the left

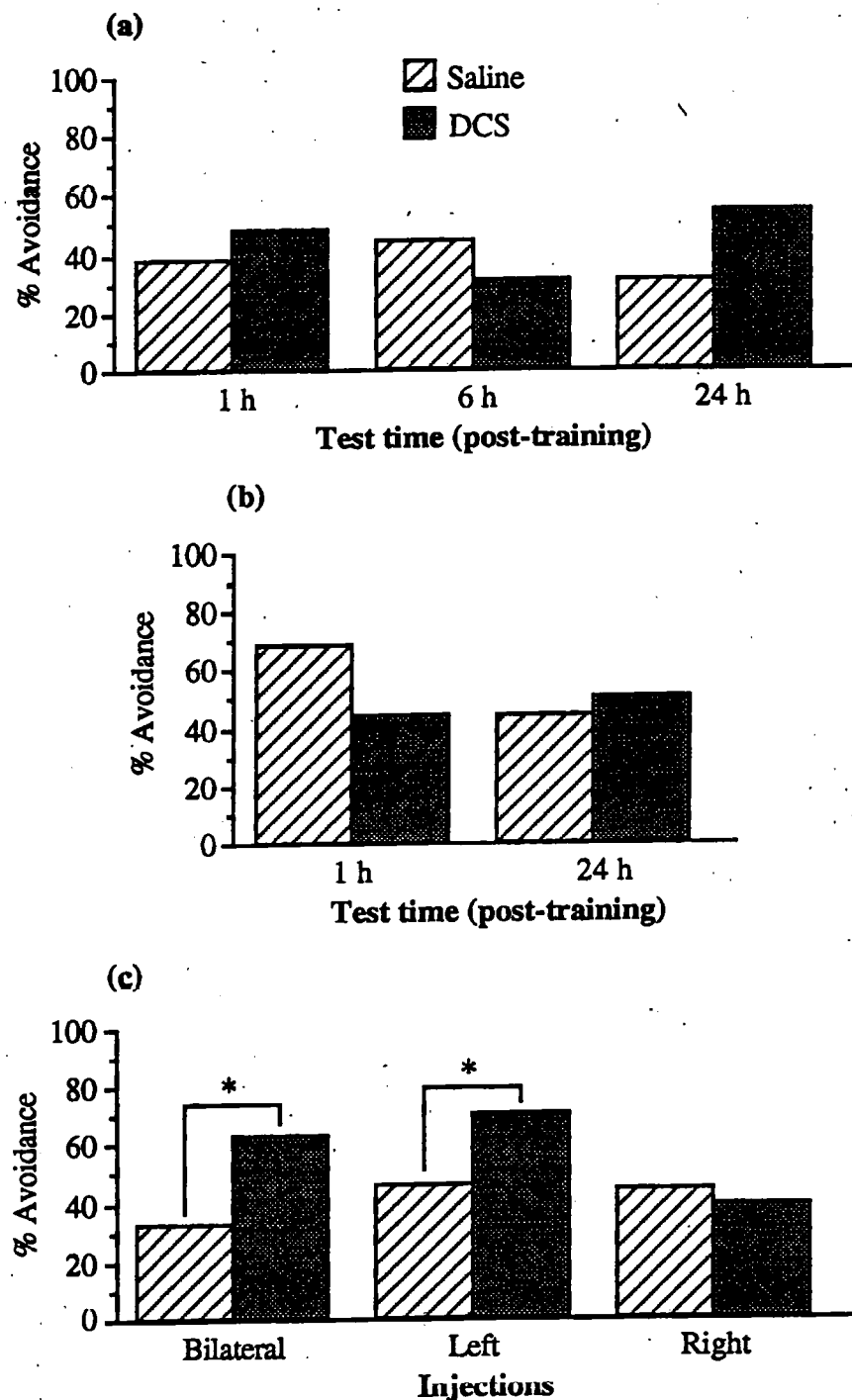


Figure 16: % avoidance following training on quinine, of chicks injected with $600 \mu\text{mol.l}^{-1}$ DCS (in 0.9% sterile saline) or 0.9 % saline (a) 10 min pre-training or (b) & (c) 5 min post-training, and tested either (a) & (b) post-training as shown, or (c) 6 h post training. The number of chicks tested in each experiment are shown in table 5. * indicates $p < 0.05$.

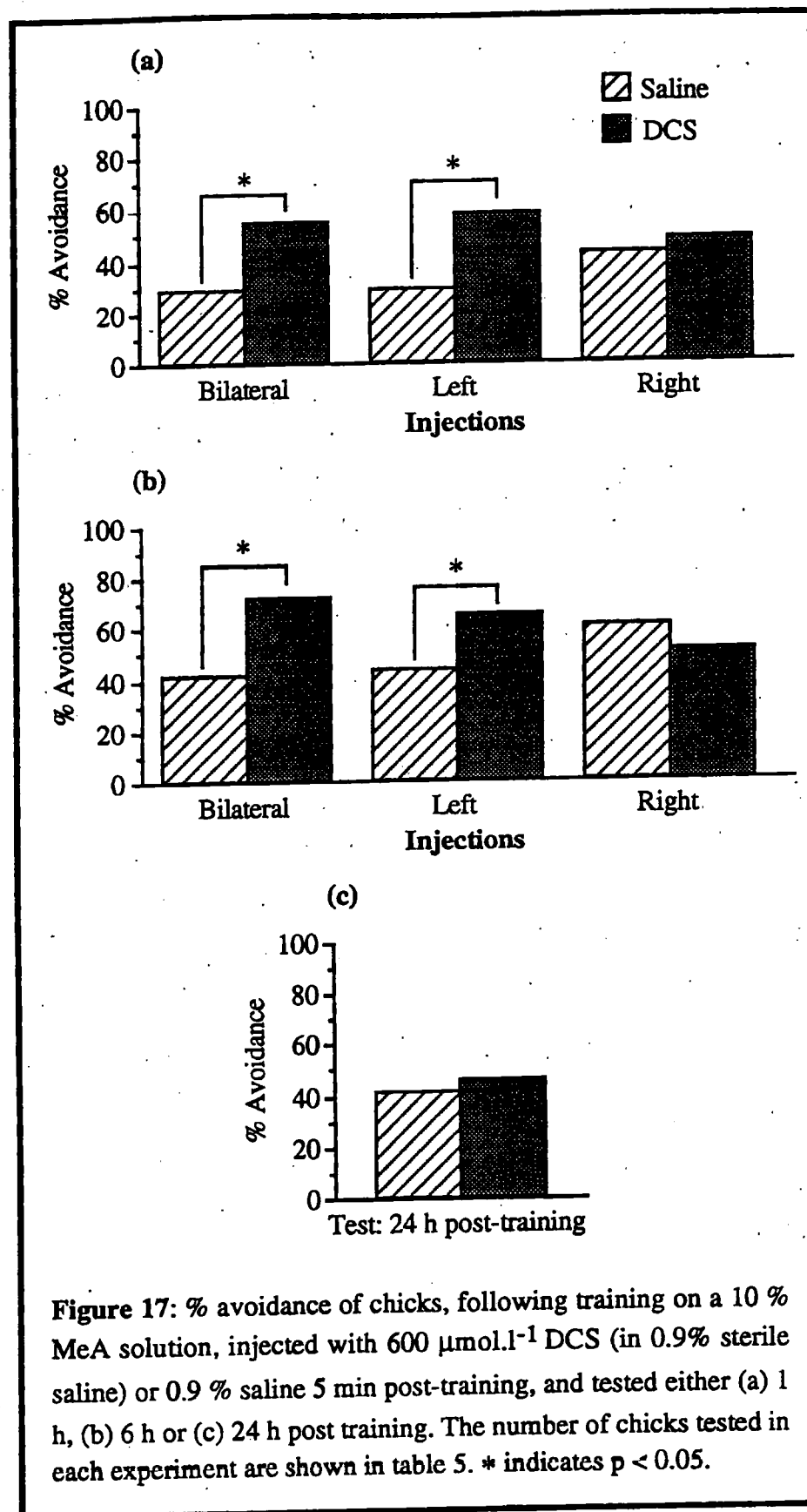
IMHV only, at this time point, a similar significant increase in avoidance occurred, but no such effect was observed with right hemisphere injections (Figure 17 (b) - left: saline - 42.8 % avoidance, DCS - 65 % avoidance, $\chi^2 = 4.91$, $p < 0.05$; right: saline - 60 % avoidance; DCS - 50 % avoidance, $\chi^2 = 0.4$).

No significant increase in avoidance occurred in DCS, compared to saline, chicks tested 24 h post-training (figure 17, c): Saline - 41.7 % avoidance, DCS - 45.8 % avoidance, $\chi^2 = 0.08$.

9.3.4: Discussion

The only obvious difference between the results obtained using quinine or 10 % MeA as the weak aversant was that, where quinine was used, 5 min post-training injections of DCS did not improve memory for the passive avoidance task 1 h post-training, but use of DCS did result in such an improvement when chicks were trained on 10 % MeA. This was largely due to the unusually high percentage of saline injected chicks which avoided the test bead 1 h after training on quinine. Training on quinine does not produce as reliable avoidance as 10% MeA, because there tends to be a relatively wide variation in responses of individual chicks. During the experiments described above, some chicks did not find it at all aversive and did not exhibit a disgust response, while others showed a disgust response which appeared to be as strong as that normally observed using 100 % MeA.

These data indicate that pre-training injections of DCS have no effect on memory for passive avoidance training on a weakly aversive stimulus. Injections given 5 min post-training, however, result in a significantly higher number of chicks which show recall up to 6 h post-training, although this effect disappears by 24 h post-training. One possible explanation for this apparent enhancement of memory by DCS, is that it actually results from non-specific inhibition of pecking behaviour. This seems unlikely, however, as the DCS chicks did not show any alteration in motor behaviour, and in the preliminary experiments performed to determine a suitable dose of DCS (section 9.3.1) no qualitative differences in



pecking ability were observed between saline and DCS injected chicks. Moreover, 5 min post-training DCS injections had no significant effect on avoidance, and therefore, no

apparent effect on pecking behaviour, where chicks were tested 1 h after training on quinine or 24 h after training with either quinine or 10 % MeA.

Assuming that increased avoidance due to DCS genuinely results from improved memory formation, these data suggest that increased activation of NMDA receptors at the time point where the consolidation of memory begins (approximately 5 min post-training^{157,180}), significantly improves the chances that, at least, the earlier stages of memory formation (up to 6 h) will occur. Furthermore, increases in the number of chicks showing recall were apparent only where chicks were injected in the left IMHV, as would be expected since significant amnesia for passive avoidance training only occurred where chicks were injected in the left IMHV with 7-ClK. Unlike 7-ClK, however, only DCS injections at 5 min post-training, and not pre-training, resulted in improved memory formation. This indicates that DCS is effective in enhancing memory formation only when it is injected close to the time when NMDA receptor activation is required to initiate processes associated with memory formation.

9.4

The NMDA receptor and its glycine co-agonist site in memory formation: general discussion.

The results discussed in this chapter confirm that NMDA receptor activation, particularly in the left IMHV, is necessary in the early stages of memory formation after passive avoidance training. Activation of the NMDA receptor's glycine site would appear to be intrinsic to its role in the initiation of memory formation, but increased activation of the glycine site alone has only a transient effect (at least on a weak aversive stimulus), significantly improving memory formation up to 6 h post training, but not at 24 h post-training. Significant increases in the release of both L-glutamate and glycine in the left IMHV, 30 min after passive avoidance training⁵³, are in accordance with the results discussed here, as are other processes which have been shown to be necessary in the early stages of memory formation, such as synthesis of the early retrograde messenger NO and

Ca^{2+} flux through N-type VGCCs. Inhibitors of NO synthase activity or of N-type VGCCs, however, result in amnesia when injected into either the right or left IMHV^{40,89}. This would suggest that processes associated with the early stages of memory formation in the right IMHV may even be initiated by some mechanism other than that involving NMDA receptors, although amnesia does become apparent 3 h post-training after MK-801 injections in the right IMHV³⁵. Nevertheless it is clear, considering the data presented in this chapter, that activation of NMDA receptors in the left IMHV, around 5 min post-training, and presumably an influx of Ca^{2+} , is a crucial initial step in converting a transient electrochemical representation of the passive avoidance task into a more durable form. Such increases in Ca^{2+} entry into neurons are known to induce processes similar to those involved in the learning and memory associated biochemical cascade²¹ (chapter 1, section 3.3 and figure 2) which eventually leads to synaptic remodelling, resulting in increased synaptic efficacy and specificity.

One of the processes involved in the early stages of memory formation, which may result from processes in the cascade, is up-regulation of NMDA receptors (sections 8.1 and 8.2). The nature of this up-regulation and the possibility of regional variation in the NMDA receptor subtypes involved in memory formation, were investigated in the experiments reported in chapter 5.

Chapter 5

NMDA receptor regulation and memory for passive avoidance training

10

NMDA receptors in memory for passive avoidance training: quantitative autoradiographic studies.

The data presented earlier provide strong evidence for the involvement of NMDA receptors in the left IMHV in the early stages of memory formation for passive avoidance training. In the chick forebrain, there are passive avoidance training related increases in binding to NMDA receptors in the left LPO and left IMHV 30 min post-training²⁰⁵ (and in the hippocampus 3 h post-training, section 8). These alterations in binding could indicate either increased synaptic efficacy, resulting from altered receptor affinity or increased numbers of receptors at individual synapses, or even increases in the number of synapses present in a region. Although preliminary data (not shown) suggest that up-regulation results from increased receptor numbers, it is important to clarify its nature more rigorously.

Some of the data from previous experiments, eg, increased [³H]MK-801 (section 8.1), but not NMDA sensitive [³H]L-glutamate binding in the right IMHV 30 min post-training, suggests the possibility that NMDA receptors in the chick brain may comprise more than one subtype. If one considers the similarity between the recently cloned avian NMDAR1 subunit and that of the rat¹⁰⁸, it is possible that avian NMDA receptors have a multiplicity of subtypes similar to that in the mammals^{136,189} (chapter 1, section 2.1.2). The experiments described in sections 10.1 and 10.2 investigate this possibility by considering binding distribution, binding alterations, and equilibrium binding kinetics of two putative

chick NMDA receptor subtypes, which may be similar to the rat NMDA preferring (nNMDA) and CPP preferring (cNMDA) subtypes (section 2.1.2).

The data presented in sections 8.1 and 8.2 showed that there is a temporal shift in the regions involved in memory for the passive avoidance task between 30 min and 3 h post-training. The experiment reported in section 10.3 will examine whether there is a further temporal shift at a later time point (6.5 h post-training).

10.1

Specificity to memory formation of increases in binding to NMDA receptors 30 min after passive avoidance training.

Introduction: The validity of presenting a water coated bead to chicks as a control for the aversive stimulus, MeA, in passive avoidance training, may be criticised on the ground that water is likely to be an appetitive stimulus and the chicks may learn something when they peck at such a control bead. An additional control (which has been used previously^{157,180}) was performed, in which chicks rendered amnesic by subconvulsive transcranial electro-shock after training, were compared to identically treated chicks which remembered to avoid the bead. In this experiment CPP sensitive [³H]L-glutamate binding was also performed and 20 forebrain regions were measured, so that the distributions of NMDA and CPP sensitive [³H]L-glutamate binding could be compared.

10.1.1: *Electro-shock and training methods*

Day old chicks were reared, housed and trained under the conditions described in chapter 2 (section 5.1). 66 chicks were placed, in pairs, in 33 pens and after an acclimatization period of 1 h were pre-trained by presenting a small white bead for 10 s, three times at 5 min intervals. Three chicks were discarded after failing to peck more than once on pre-training. 10 min after the third pre-training trial, chicks were presented with a chrome bead coated with either MeA or water. 10 pairs of chicks were presented with the W

bead and 10 pairs with the MeA bead. Chicks were tested 30 min after training, using a similar but dry chrome bead and only those giving the appropriate response, that is avoiding if MeA trained or pecking if W control (~85% of chicks), were used for further experimentation (1 chick from each of 18 pens, giving 9 W and 9 MeA chicks). A subconvulsive transcranial electro-shock (12 mA, 110 V, 220 ms duration at 50 Hz) was administered to the 23 remaining chicks (see acknowledgements), 5 min post-training on the MeA bead. When electro-shock is administered immediately after training, chicks become amnesic and peck the bead on re-testing. When the shock is delayed until 10 min post-training chicks show recall and remember to avoid the bead on re-testing¹⁸⁰. When electro-shock is administered 5 min post-training, however, roughly half remember and half are amnesic¹⁵⁷. In this experiment, when chicks were tested 30 min after training, 13 chicks pecked (electro-shock amnesia - ESA), and 10 chicks avoided (electro-shock recall - ESR). Nine ESA and nine ESR chicks were selected for further experimentation. Immediately after testing, chicks were killed by decapitation and the brains were removed from the skull, dissected and frozen as described in chapter 2 (section 5.3.1). Brains were coded and during all subsequent procedures the experimental history of the chicks was unknown to the researcher.

10 μ m coronal sections were cut from two locations of the chick forebrain (equivalent to those shown in figure 3, b and d, chapter 1), thaw mounted onto coverslips and stored, as described in chapter 2 (section 5.3.2). These locations are referred to as anterior (fig 3, b) and intermediate (fig 3, d) forebrain. These particular locations were carefully chosen so that the LPO (anterior) and the IMHV (intermediate) could be identified easily, and they correspond to levels 1 and 3 respectively in figure 3 (a). The intermediate sections were cut slightly posterior to those discussed in chapter 3 (section 8), so that the AIv and AI_d could be measured rather than the AA. Fifteen sections were cut from each location of each of the 36 brains. Nine sections were selected from each location of each brain for the experiment (324 anterior and 324 intermediate sections in all) and the others were used for scintillation counting and test strips (see chapter 1 sections 5.3.4 and 5.3.6).

The sections were brought to room temperature and divided appropriately between numbered racks, before being pre-incubated in TCB at 4°C for 1 h, to remove endogenous ligands.

10.1.2: Incubations and densitometry

Sections from anterior and intermediate brain locations were pre-incubated and incubated in separate assays, on consecutive days. In each assay sections were arranged appropriately on aluminium sheets, on ice and incubations were performed as described in chapter 2 (section 5.3.3). Three sections from each of the 36 brains, plus six spare sections for preparation of autoradiographic test strips and scintillation counting, were incubated for 20 min with either 100 nmol l⁻¹ [³H]L-glutamate in TCB alone, or with 1 mmol l⁻¹ NMDA or 1 mmol l⁻¹ CPP. This gave 36 triplicate sections (one triplicate per brain, 9 brains per training group, W, MeA, ESA and ESR), plus 6 spare sections, for total binding. Identical numbers of sections were used for NSB after NMDA displacement, and for NSB after CPP displacement at both anterior and intermediate locations. Sections were washed 3 times in TCB (10 s each) to terminate each incubation, dip washed in distilled water to remove buffer salts and fan dried. Three lots of triplicate spare sections from each location, to which each of the three incubation solutions were applied, were selected for scintillation counting and the remaining spare sections were used for test strips (see section 5.3.6). 25 µl of the [³H]L-glutamate solution applied to the sections, was also placed in each of three scintillation vials, and counted so that the accurate concentration could be determined (section 5.3.4). These were 71.1 nmol l⁻¹ for anterior sections and 73.5 nmol l⁻¹ for intermediate sections, specific activity = 108.8 dpm/fmol. The average activities of counted sections were, for anterior: total binding = 520 dpm, NMDA displaced = 291 dpm, CPP displaced = 240 dpm, and for intermediate: total binding = 700 dpm, NMDA displaced = 403 dpm, CPP displaced = 352 dpm. This indicates that NMDA and CPP both displaced appreciable amounts of [³H]L-glutamate and although CPP displaced more than NMDA the difference was not significant.

Sections were apposed to [³H]Ultrafilm (section 5.3.6), with pre-prepared brain paste standards (section 5.3.5). Sections were apposed to six sheets of film (108 sections,

or 36 triplicates per film) for up to 61 days, as indicated by activity bound to spare sections²¹⁹, and the apposition time required for test strips. Autoradiograms were developed as described in section 5.3.6

Densitometry of autoradiograms was performed on a Joyce-Loebl Magiscan MD image analysis system, as described in chapter 2 section 5.3.7. The binding density of NMDA and CPP sensitive [³H]L-glutamate binding in 20 forebrain regions (10 regions per brain location) were measured separately in each hemisphere, in addition to whole section and hemisphere measurements. These are the labelled regions in figure 3 (a and b) (chapter 1). The data were processed using Apple Macintosh software (see chapter 2, section 6). Background readings from the image analysis system were subtracted, triplicate means were taken, and data were sorted by region. Non-specific binding data (for both NMDA and CPP as displacer) were subtracted and data were standardized as described in chapter 2 section 5.3.7. All measurements and operations on data, until this point, were performed blind. Finally the data were sorted by code into MeA, W, ESR and ESA groups. This gave four sets of nine values (W and MeA) for each region measured in each hemisphere, and for each of NMDA and CPP sensitive [³H]L-glutamate binding. The means, standard deviations and standard errors of the mean for these were then calculated.

Analyses of regional binding differences between NMDA and CPP sensitive [³H]L-glutamate, W and MeA chicks, and between ESA and ESR chicks were performed using a two way analysis of variance (ANOVA) with a split plot design and Students t-tests, to find whether any of the differences were statistically significant. These analyses were performed using statistical functions on Microsoft Excel 4 worksheets.

10.1.3: Results: regional binding differences between NMDA and CPP sensitive [³H]L-glutamate

Examples of the distribution of [³H]L-glutamate binding, and that of its displacement by NMDA and CPP, to typical sections are shown in Figure 18 (c- h) (see also table 7). The

binding distribution of [^3H]L-glutamate and its displacement by NMDA is similar both to that described in chapter 3 (section 8.1.2 and figure 10), and as reported by Henley et al (1989)⁸⁴. Figure 18 shows that in most regions, the degree of displacement by NMDA and CPP is very similar. Closer observation, however reveals that in a few regions there are

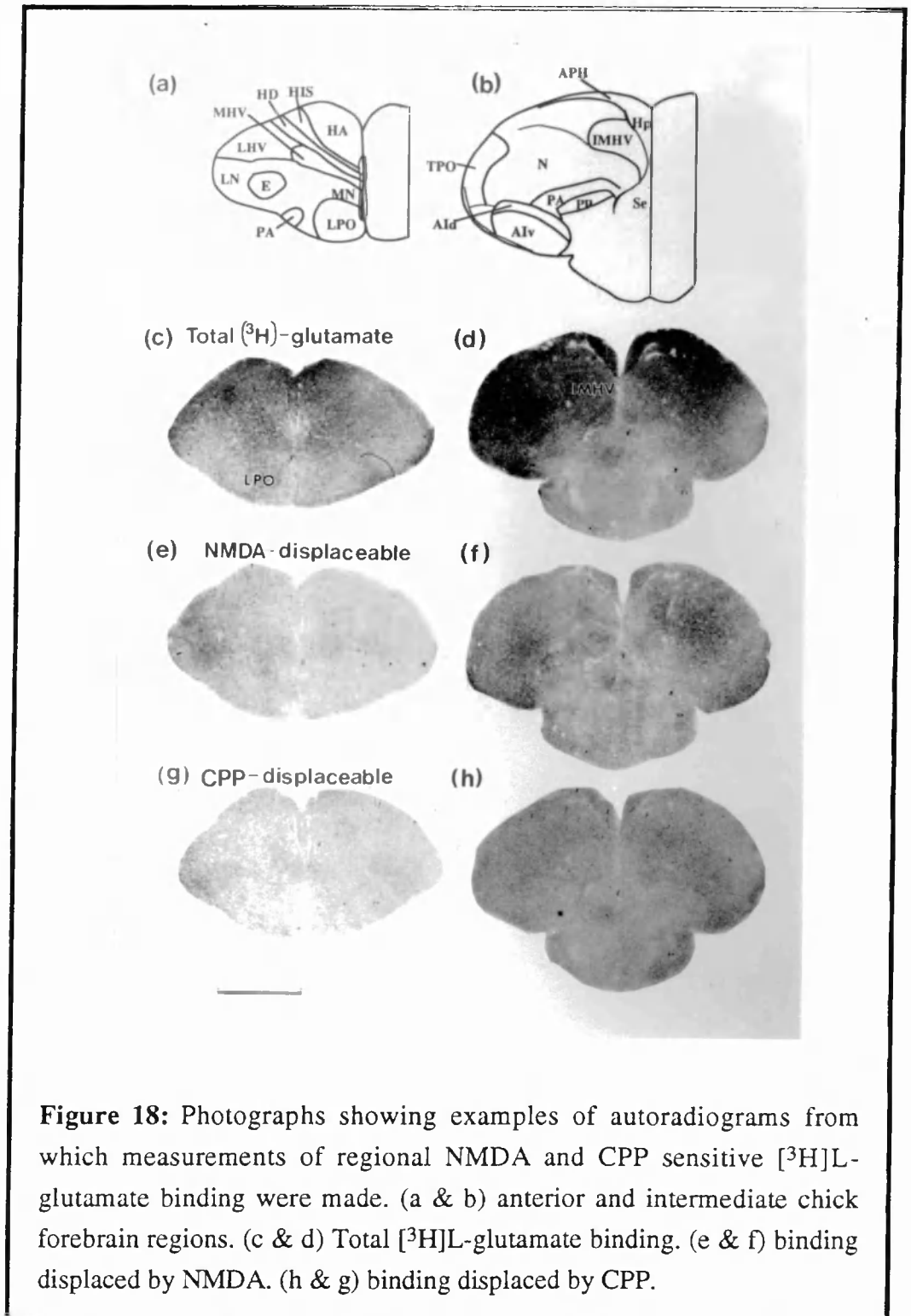


Figure 18: Photographs showing examples of autoradiograms from which measurements of regional NMDA and CPP sensitive [^3H]L-glutamate binding were made. (a & b) anterior and intermediate chick forebrain regions. (c & d) Total [^3H]L-glutamate binding. (e & f) binding displaced by NMDA. (h & g) binding displaced by CPP.

Table 7: Data for binding of radioligands to coronal chick forebrain sections, 30 min after passive avoidance training. W = water trained, MeA = MeA trained, ESA = E-shock amnesic, ESR = E-shock recall.

Binding Of:			NMDA sensitive [³ H]glutamate					
Condition	Control (W)		Trained (MeA)		Control (ESA)		Trained (ESR)	
Hemisphere	Left	Right	Left	Right	Left	Right	Left	Right
(a) Anterior brain regions								
LPO	<u>*11.2±0.5</u>	<u>16.7±1.1</u>	*19.1±1.9	15.6±2.2	*12.3±1.8	16.1±2.1	*22.2±1.4	13.9±0.8
PA	18.6±1.7	19.3±2.1	17.2±2.1	17.3±1.8	20.8±1.9	20.7±1.8	17.1±1.3	17.4±1.1
E	-0.3±1.7	1.1±1.8	1.0±2.2	1.2±1.3	0.1±1.8	-0.6±0.7	-0.7±0.4	-0.4±0.5
MN	14.5±1.0	14.1±0.9	15.4±1.2	13.2±0.9	13.6±0.8	12.0±1.4	13.9±0.3	12.0±1.0
LN	18.8±1.6	16.9±2.2	23.1±2.8	14.6±1.8	18.0±1.9	12.6±1.3	16.9±1.7	13.4±1.0
MHV	<u>23.9±1.7</u>	<u>23.5±2.4</u>	25.5±3.2	23.3±2.3	24.5±2.7	27.3±2.4	27.1±2.5	27.1±2.3
LHV	18.3±1.7	22.1±0.9	22.1±0.9	15.1±1.7	17.4±1.0	20.1±0.9	21.7±2.9	17.2±1.6
HD	14.5±1.0	14.1±1.5	13.0±1.1	11.9±1.0	14.5±1.1	13.5±0.9	16.7±0.8	14.5±1.0
HIS	13.4±1.5	12.6±1.3	16.7±1.2	14.2±0.8	16.5±1.7	16.7±0.8	12.8±1.1	12.8±1.1
HA	17.8±1.2	18.0±1.6	17.8±1.5	18.4±1.6	21.9±1.6	21.7±1.6	19.6±1.5	18.2±1.4
(b) Intermediate brain regions								
AIv	12.4±0.7	16.2±1.0	12.7±1.4	13.1±2.7	11.2±0.4	13.3±1.5	12.0±1.3	14.4±1.2
AId	8.9±0.8	10.2±2.1	8.6±0.7	9.2±0.5	8.2±0.7	8.0±0.7	8.2±0.6	8.4±0.3
PP	9.8±0.4	9.4±0.5	9.3±0.5	8.9±0.5	7.6±0.4	8.3±0.7	9.2±0.8	9.4±0.5
PA	<u>14.7±1.0</u>	<u>9.2±0.5</u>	12.6±0.7	12.6±1.0	14.7±1.2	14.8±0.6	13.9±1.4	14.7±1.7
Se	7.9±0.7	7.5±0.6	7.7±0.5	7.8±0.6	7.4±0.4	7.0±0.3	7.8±0.5	7.6±0.6
N	16.3±1.5	15.2±1.8	14.4±1.6	14.2±1.4	17.6±2.7	17.6±1.7	17.4±2.5	16.7±2.2
TPO	13.3±1.8	13.2±1.7	12.9±2.9	13.4±1.8	13.1±1.6	13.5±1.9	10.7±1.8	13.1±1.6
IMHV	<u>*11.0±1.1</u>	<u>16.2±1.4</u>	*16.5±1.6	13.2±1.0	*11.1±1.3	15.6±1.0	*18.4±2.4	13.2±1.6
Hp	14.6±1.8	14.1±1.0	15.2±1.9	14.1±1.7	14.7±1.1	14.4±1.5	17.8±1.8	16.8±1.3
APH	16.6±2.4	15.3±1.4	14.1±2.3	11.4±1.1	16.5±1.6	13.8±2.1	14.0±1.1	14.9±1.1

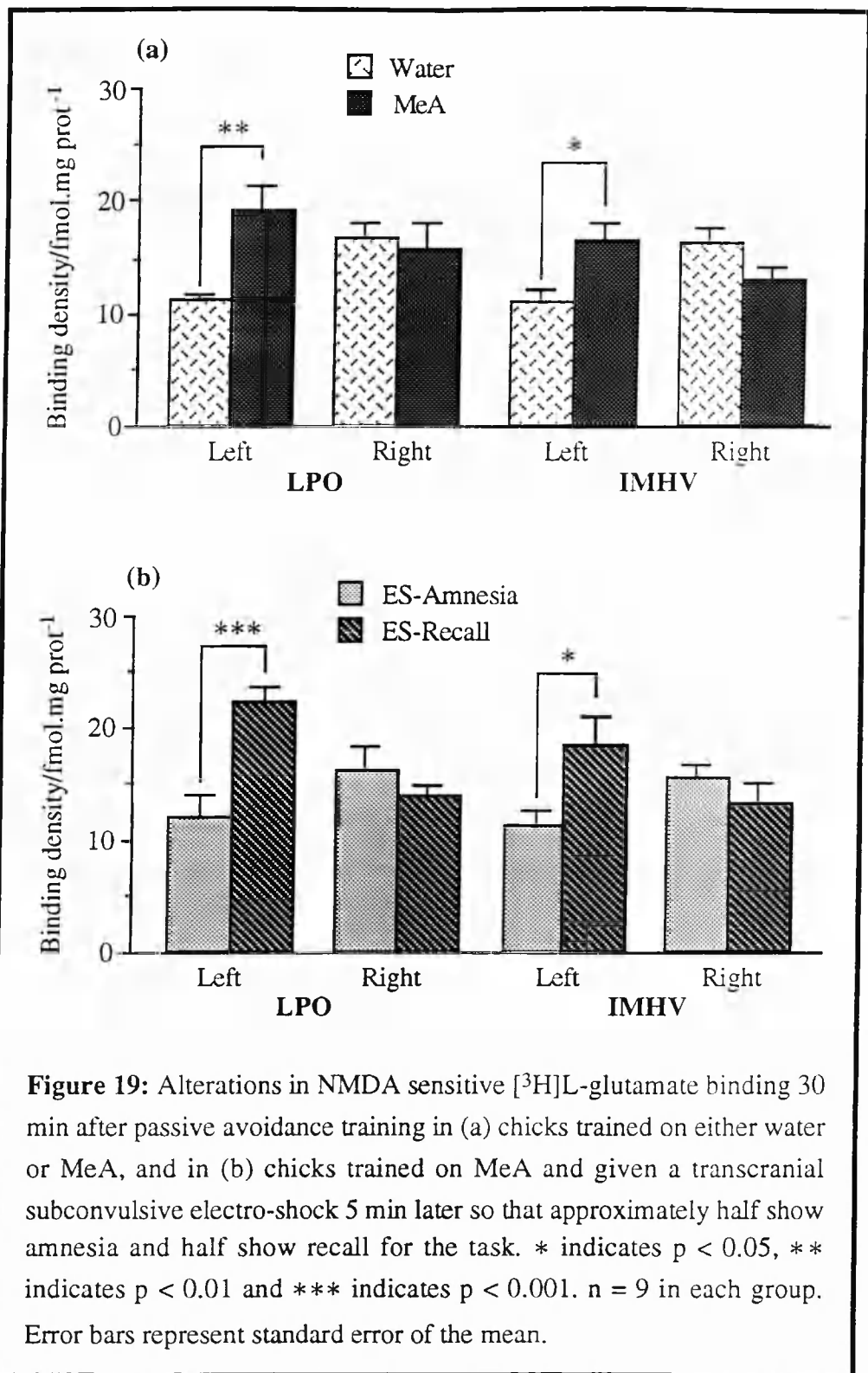
Binding Of:			CPP sensitive [³ H]glutamate					
Condition	Control (W)		Trained (MeA)		Control (ESA)		Trained (ESR)	
Hemisphere	Left	Right	Left	Right	Left	Right	Left	Right
(a) Anterior brain regions								
LPO	<u>10.6±0.5</u>	<u>11.2±1.3</u>	14.1±1.4	10.8±1.1	11.1±2.1	12.6±1.5	15.3±1.2	11.1±1.3
PA	17.4±1.6	19.1±2.1	16.3±2.3	17.8±1.8	20.4±1.8	20.3±1.5	17.1±0.9	17.5±1.4
E	2.4±1.6	3.2±1.5	2.9±1.6	2.6±1.1	1.3±1.5	0.1±2.2	1.7±0.9	3.3±1.4
MN	9.2±1.2	7.5±0.8	9.7±0.9	8.1±0.5	8.1±0.8	5.7±1.2	8.5±0.6	7.3±0.7
LN	15.7±2.0	16.4±1.9	19.9±2.5	14.6±1.4	14.7±2.5	12.5±1.3	14.9±1.6	13.5±1.0
MHV	<u>18.5±1.4</u>	<u>17.8±2.5</u>	20.1±2.6	17.9±1.6	18.7±2.2	21.2±1.9	21.1±1.6	21.1±1.8
LHV	15.2±1.7	12.5±1.6	18.5±1.5	13.0±1.3	14.1±1.4	17.7±0.7	19.7±3.2	15.3±1.7
HD	11.2±1.0	11.3±1.4	11.1±1.0	9.8±1.0	12.1±1.2	10.3±1.1	13.5±0.9	11.7±1.1
HIS	9.7±1.4	8.7±1.1	12.6±0.8	11.1±0.7	12.3±1.8	13.8±1.4	9.8±1.4	9.5±0.9
HA	15.1±1.5	15.4±1.7	15.6±1.4	16.0±1.8	20.2±1.7	21.0±1.7	17.4±1.3	16.3±1.3
(b) Intermediate brain regions								
AIv	14.1±0.7	17.8±1.0	13.9±1.6	15.2±2.8	12.0±0.6	14.3±1.5	12.8±1.6	16.3±1.4
AId	9.9±0.7	10.4±0.6	9.7±0.7	10.6±0.6	8.7±0.6	8.9±0.5	9.0±0.7	9.9±0.5
PP	10.7±0.4	10.2±0.5	10.0±0.5	9.7±0.4	9.1±0.4	9.5±0.6	10.3±0.9	10.5±0.6
PA	<u>17.2±1.1</u>	<u>17.2±0.9</u>	15.8±1.0	14.7±0.4	16.5±1.0	16.7±0.7	16.3±1.3	17.1±1.9
Se	9.0±0.7	8.5±0.7	8.5±0.7	8.4±0.7	8.3±0.4	8.2±0.3	8.5±0.4	8.5±0.5
N	16.3±1.3	15.4±1.4	14.7±1.3	16.8±1.6	21.0±2.2	21.3±2.0	21.2±2.5	19.7±2.3
TPO	14.1±1.7	14.0±1.7	15.0±3.3	13.8±2.0	14.7±1.5	14.2±1.8	12.8±2.0	14.2±1.6
IMHV	<u>9.1±0.7</u>	<u>11.5±0.9</u>	12.0±1.3	9.8±1.1	13.9±1.1	16.5±0.9	18.9±2.4	14.8±2.3
Hp	12.3±2.0	10.7±1.6	16.0±1.7	15.5±1.6	15.0±1.4	*14.6±1.7	19.9±1.7	*18.9±1.4
APH	16.7±2.7	15.9±1.7	15.9±3.0	12.5±1.2	17.7±1.5	14.5±1.9	15.0±1.2	15.5±1.0

Values were calculated in fmol.mg prot⁻¹ ± standard error of the mean. n = 9 for each group. Significant differences between control and test conditions are indicated by asterisks. and significant differences between NMDA and CPP displacement are underlined (see figs 18 - 20)

visible differences. Comparison of the autoradiograms in figure 18 (f and h) shows that in the IMHV, NMDA displaces [^3H]L-glutamate more effectively than does CPP (the IMHV is lighter relative to surrounding regions in f than in h). The opposite is true in the AIv, the PA and the ectostriatum, where CPP is a more effective displacer than NMDA. This is particularly the case in the PA. It can be clearly seen in figure 18 (f) that the PA is appreciably darker than surrounding regions, whereas in figure 18 (h) it is lighter than surrounding regions. These visible differences between NMDA and CPP displacement were tested statistically in W control chicks, along with other differences in binding which were obvious from the data, but are less obvious qualitatively on the autoradiograms in figure 18. In anterior sections, NMDA displaced more [^3H]L-glutamate than CPP, but in intermediate sections CPP displaced 13 % more [^3H]L-glutamate than NMDA. The differences between NMDA and CPP displacement of [^3H]L-glutamate in the ectostriatum (CPP > NMDA by 61 %) and AIv (CPP > NMDA by 10.5 %) were non-significant. Differences between NMDA and CPP displacement of [^3H]L-glutamate, however, proved to be significant in the IMHV (NMDA > CPP by 24 %, $F_{1,16} = 5.59$, $p = 0.031$), anterior MHV (NMDA > CPP by 23.4%, $F_{1,16} = 4.9$, $p = 0.048$), PA (CPP > NMDA by 13.5 %, $F_{1,16} = 4.76$, $p = 0.044$) and LPO (NMDA > CPP by 21.7 %, $F_{1,16} = 6.97$, $p = 0.018$).

10.1.4: Results: memory specific alterations in NMDA sensitive [^3H]L-glutamate binding

Data from this experiment are shown in figures 19 (a and b). There were no significant differences between either MeA and W chicks, or between ESR and ESA chicks, except in the LPO and IMHV. ANOVA of data from the LPO of either MeA compared to W chicks, or ESR compared to ESA chicks, shows that the effect of training on NMDA sensitive binding is significant in both cases (figure 19 (a), W vs MeA: $F_{1,16} = 7.0$, $p = 0.017$; figure 19 (b), ESA vs ESR: $F_{1,16} = 5.5$, $P = 0.032$). In each case t-tests also indicated significant increases in binding in the left hemisphere (figure 19 (a), W vs MeA: 70.9%, $t = 3.94$, $df = 16$, $p = 0.001$; figure 19 (b), ESA vs ESR: 80.9%, $t = 4.39$, $df = 16$, $p < 0.0005$).

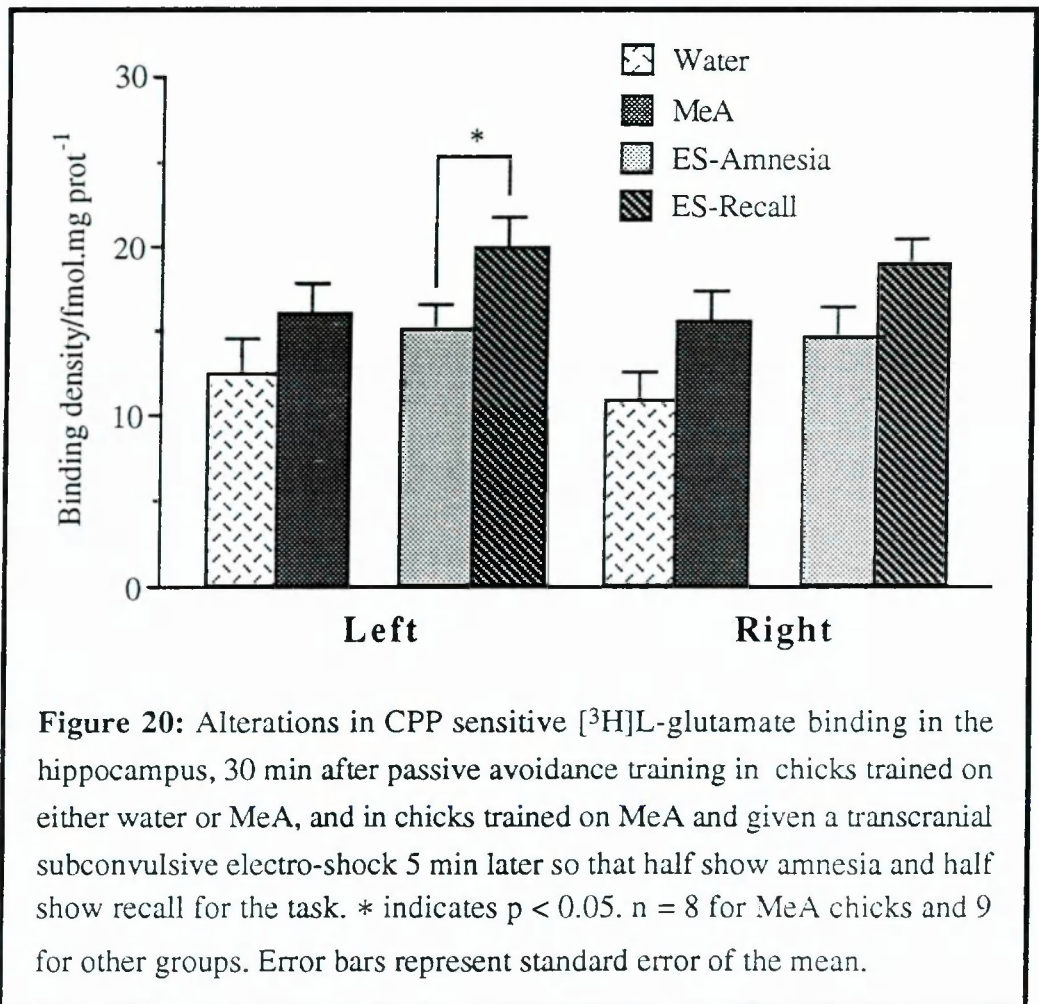


ANOVA of data from the IMHV revealed that the interaction of training and hemisphere factors was significant too for MeA vs W or ESA vs ESR (figure 19 (a), W vs MeA: $F_{1,11} = 166.5$, $p = 0.049$; figure 19 (b), ESA vs ESR: $F_{1,11} = 211.2$, $p = 0.044$). This was due to left hemisphere increases in binding coupled with decreases in binding in the right hemisphere.

T-tests indicated that the left hemisphere increases in binding were significant (figure 19 (a), W vs MeA: 50.8% , $t = 2.88$, $df = 16$, $p < 0.011$; figure 19 (b), ESA vs ESR: 65.5%, $t = 2.67$, $df = 16$, $p < 0.017$).

10.1.5: Results: Memory specific alterations in CPP sensitive [^3H]L-glutamate binding

The only significant change in CPP sensitive [^3H]L-glutamate binding revealed by ANOVA was in the hippocampus of ESR compared to ESA chicks ($F_{1,16} = 4.77$, $p = 0.044$). t-tests revealed this to be due to a significant increase in binding to the left hemisphere (figure 20: 32.5%, $t = 2.19$, $df = 16$, $p = 0.044$). A similar increase in binding in the right hemisphere (29.5%) was non-significant. Although no significant increases in CPP sensitive



binding occurred in MeA compared to W chicks, non-significant increases in the hippocampus, similar to those above were apparent (Left: 29.7%, Right: 44.6%), and that in the right hemisphere was in fact very close to significance ($F_{1,15} = 3.05$, $p = 0.1$, $t = 2.12$, $p = 0.0502$).

10.1.6: Discussion

The data presented in figure 19 (a) reveal alterations in NMDA sensitive [^3H]L-glutamate binding similar to those reported in our previous study where chicks were killed 30 min post-training (section 8.1)²⁰⁵, with significant increases in the left LPO and in the left IMHV. The decrease in binding to the right LN reported in section 8.1, however, was not observed here. The observed increases in NMDA sensitive binding in the left LPO and left IMHV of ESR compared to ESA chicks (figure 19, b), were slightly greater than those observed in the same brain regions of the MeA compared to water chicks (figure 19, a), but not significantly so. No other significant binding alterations were observed under either training condition. These findings strongly suggest that the increases in NMDA sensitive binding in ESR, compared to ESA, chicks can be specifically attributed to biochemical processes associated with memory formation, and not to any concomitants, such as the perception of the unpleasant taste of MeA. The similarity in binding alterations between the two training/control conditions (MeA/W, ESR/ESA) also indicates that the appetitive nature of the water coated control bead does not appear to have a significant effect on the observed regional changes in NMDA sensitive [^3H]L-glutamate binding, under the conditions used, validating the results of other such experiments where the water control has been used.

None of the training related alterations in NMDA sensitive [^3H]L-glutamate binding were observed when CPP sensitive [^3H]L-glutamate was examined, and the only training related alterations in CPP sensitive binding occurred in the hippocampus, a region where there were little or no changes in NMDA sensitive binding. The only significant increase in CPP sensitive binding was in the left hippocampus of the electro-shocked chicks. Those in the right hippocampus of both MeA and ESR chicks, however, were sufficiently large to

indicate a trend towards bilateral increases in binding in this region (figure 20). The results of a preliminary study of the equilibrium binding kinetics of CPP sensitive [^3H]L-glutamate binding (data not shown), suggest that increased CPP sensitive binding in the hippocampus 30 min post-training may be due to increased receptor affinity. This preliminary work also showed a large increase in CPP sensitive binding in the right IMHV, however, which was not observed in this study. These preliminary findings must, therefore be treated with extreme caution.

Differences in binding distribution between NMDA and CPP in the rat brain have been cited as evidence for the existence of two or more, pharmacologically distinguishable NMDA receptor subtypes^{34,136}. The evidence from this experiment, ie, significant differences between NMDA and CPP sensitive [^3H]L-glutamate binding in the IMHV, LPO and PA, indicates that this may also be the case in the chick brain. In addition, their differential pattern of training related binding alterations suggests that there may be regional differences in the roles of at least two NMDA receptor subtypes in memory formation. Further investigation of this, in addition to the nature of NMDA receptor up-regulation is reported in sections 10.2 and 10.3.

10.2

Alterations in the equilibrium binding parameters of NMDA receptors, 3 h after passive avoidance training.

Introduction: In this experiment the effect of passive avoidance training on the equilibrium binding parameters of NMDA receptors in the chick forebrain, 3 h post-training, was investigated using NMDA and CPP sensitive [^3H]L-glutamate binding. The main aims were to confirm that previous findings of NMDA sensitive binding alterations at 3 h post-training were due to increased numbers of receptors, as suggested by preliminary kinetic data at 30 min post-training; to find whether differences in any passive avoidance related alterations of NMDA and CPP sensitive binding occur at 3 h post-training; and to confirm

the presence of at least two NMDA receptor subtypes in the chick forebrain by performing Scatchard analyses to distinguish binding components.

10.2.1: Procedure

Day old chicks were reared, housed and trained under the conditions described in chapter 2 (section 5.1). 40 chicks were placed in pairs in 20 pens, and after acclimatization were pre-trained, trained and tested using a protocol identical to that described in section 10.1.1 for W/MeA chicks, except that chicks were tested and killed 3 h post-training. Only those giving the appropriate response were used for further experimentation (1 chick from each of 18 pens, providing 9 W and 9 MeA trained). 48 sections were cut from each of the anterior and intermediate forebrain locations (figure 3, a and d), in each brain.

One triplicate and 18 sets of duplicate sections were selected from each location of each brain for NMDA and CPP sensitive [^3H]L-glutamate binding. the remaining sections were used for scintillation counting and test strips (see chapter 1 sections 5.3.4 and 5.3.6). One brain was damaged and could not be used for further experimentation.

Pre-incubations and incubations were performed as described in sections 5.3.3 and 10.1.1. NMDA and CPP sensitive [^3H]L-glutamate binding assays to anterior and intermediate sections were performed separately. Prior to the incubations, nine concentrations each (1 ml volumes) of NMDA and CPP solutions were made up by 75 % serial dilutions, (top: 200 $\mu\text{mol l}^{-1}$) to each of which, 1 ml of 200 nmol l^{-1} [^3H]L-glutamate was added, giving final displacer concentrations of 100, 25, 6.25, 1.56, 0.39, 0.098, 0.024, 0.006, 0.0015 and 0 $\mu\text{mol l}^{-1}$, all in 93.1 nmol l^{-1} [^3H]L-glutamate (accurate concentration determined by scintillation counting, s.a = 108.8 dpm/fmol). In both assays, 2.8 ml of 200 nmol l^{-1} [^3H]L-glutamate was added to 2.8 ml of TCB for total binding. 17 triplicates (1 from each brain), were incubated with [^3H]L-glutamate. Nine further groups of sections per location were incubated with the above range of NMDA concentrations in [^3H]L-glutamate, and another nine were incubated with an identical concentration range of

CPP in [^3H]L-glutamate. To terminate the incubations, all sections were washed three times in ice-cold buffer for 10 s, dipped in ice-cold distilled water to remove buffer salts and rapidly air dried.

After processing, sections were apposed to [^3H]Ultrafilm (a maximum of 51 sections per film) with pre-prepared brain paste standards (section 5.3.5) and developed as previously described (section 5.3.6). Apposition time was estimated as previously, from scintillation counts of spare sections incubated along with those for autoradiograms²¹⁹, as 60 days. Actual apposition time was 64 days. Autoradiograms were developed as described in section 5.3.6.

Densitometry of autoradiograms was performed as described in chapter 2 section 5.3.7. The binding density of NMDA and CPP sensitive [^3H]L-glutamate in eight intermediate brain regions (AIv, AI_d, PA, N, TPO, IMHV, HP and APH) were measured separately in each hemisphere, in addition to whole section and hemisphere measurements. Anterior regions were not measured due to poor binding. The data were processed as described in section 10.1.1 up to the standardization stage, except that they were calculated as dpm values for entry into EBDA. Here standardization was performed as described in section 5.3.7, but to data from binding in the presence of each displacer (or radioligand) concentration. Scatchard analyses were performed on regional binding data from each individual brain, using EBDA/LIGAND. Resulting B_{max} and K_D values were sorted by code into W and MeA groups for each region measured in each hemisphere, and for each of NMDA and CPP sensitive [^3H]L-glutamate binding. The means, standard deviations and standard errors of the mean were then calculated. The means of the decoded binding data for whole section measurements of both NMDA and CPP sensitive [^3H]L-glutamate binding (average of left and right hemisphere data) in W control chicks, were then taken and analysed using EBDA/LIGAND to find the average K_D and B_{max} values for whole sections. Regional Hill coefficients were calculated in EBDA. Analyses of regional binding

differences in B_{max} and K_D , between W and MeA chicks, were performed using ANOVA and t-tests, as in section 10.1.1.

10.2.2: Results: The parameters of NMDA and CPP sensitive [3H]L-glutamate binding in the day old chick forebrain

Analysis of average binding to whole sections from control chicks in EBDA/LIGAND, gave nH values of 0.547 ± 0.074 for NMDA sensitive binding and 0.552 ± 0.112 for CPP sensitive binding, both were significantly less than 1 and not significantly different from 0.5, indicating a high likelihood of heterogeneity between two binding sites in both cases. ANOVA performed by LIGAND during Scatchard analysis, revealed that a two site (curvilinear) model represented a significant improvement of fit compared to a one site model, for both NMDA and CPP sensitive [3H]L-glutamate binding (NMDA: $F_{6,5} = 8.94$, $p = 0.033$; CPP: $F_{6,5} = 6.26$, $p = 0.049$ for CPP). The Scatchard plots generated by this analysis, with two component curves, are shown in figure 21.

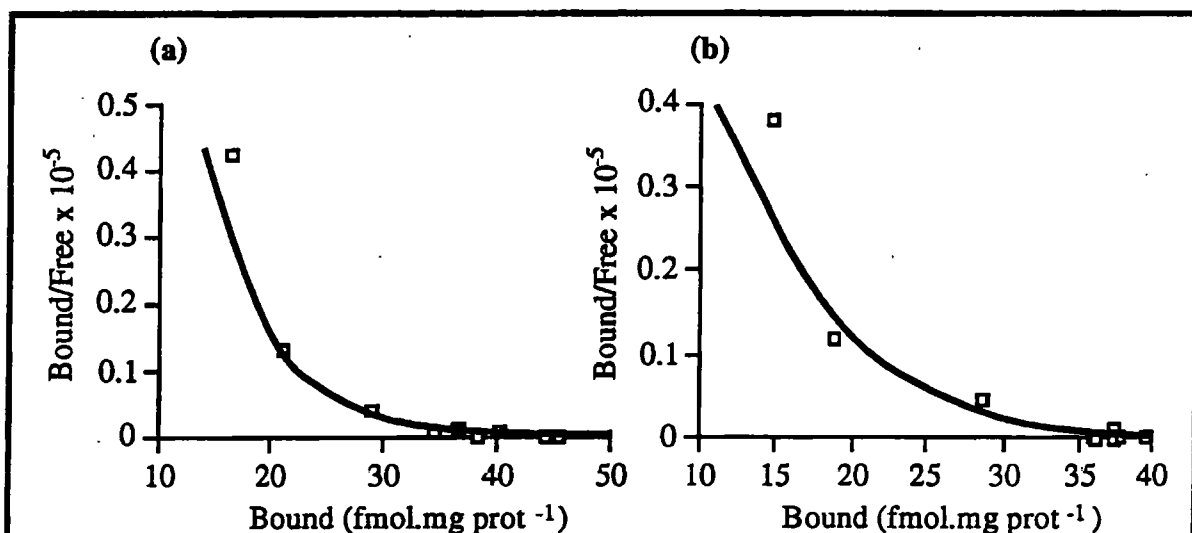


Figure 21: Scatchard plots of mean binding to whole coronal intermediate forebrain sections (figure 3, d) from control chicks ($n = 9$), generated by LIGAND, for (a) NMDA sensitive and (b) CPP sensitive [3H]L-glutamate binding, 3 h post-training. Both plots show a non-linear curve fit for a two component binding model. This represented a significant improvement of fit over that for a one component model in both cases (see text). The binding parameters (B_{max} and K_D) for these plots are given in the text.

The equilibrium parameters of binding to whole sections from control chicks, as calculated by LIGAND, for NMDA sensitive binding were; high affinity component (A_H): $K_D = 22.4 \pm 6.4 \text{ nmol l}^{-1}$, $B_{\max} = 21.92 \pm 5.94 \text{ fmol mg prot}^{-1}$; low affinity component (A_L): $K_D = 2.47 \pm 0.48 \text{ } \mu\text{mol l}^{-1}$, $B_{\max} = 54.55 \pm 7.55 \text{ fmol mg prot}^{-1}$, and for CPP sensitive binding were; A_H : $K_D = 31.20 \pm 8.2 \text{ nmol l}^{-1}$, $B_{\max} = 22.12 \pm 5.8 \text{ fmol mg prot}^{-1}$; A_L : $K_D = 0.87 \pm 0.11 \text{ } \mu\text{mol l}^{-1}$, $B_{\max} = 40.53 \pm 6.46 \text{ fmol mg prot}^{-1}$.

In control chicks, regional B_{\max} values varied with the binding distributions of NMDA and CPP sensitive [^3H]L-glutamate (Figure 18). All regional K_D values were similar to those above and no significant variation between regions was observed. A_H K_D values for CPP sensitive binding were significantly greater than those for NMDA sensitive binding in all regions, whereas the opposite was true of A_L K_D values. On average, the K_D for A_H NMDA sensitive binding was 30 % less than that for CPP sensitive binding (ANOVA: $F = 26.2$, $p = 0.0001$) and K_D for A_L NMDA sensitive binding was 65 % greater than that for CPP sensitive binding (ANOVA: $F = 9.55$, $p = 0.007$).

10.2.3: Results: Alterations in the parameters of NMDA and CPP sensitive [^3H]L-glutamate binding, 3 h after passive avoidance training

ANOVA performed on B_{\max} and K_D values, derived from Scatchard analysis of NMDA sensitive [^3H]L-glutamate binding, in the regions investigated, revealed that the effect of training (W,MeA) on both A_H and A_L B_{\max} values was significant in the hippocampus (A_H : $F_{1,15} = 11.08$, $p = 0.005$; A_L : $F_{1,13} = 5.66$, $P = 0.03$). Analyses by t-tests indicated that this was due to significant bilateral increases of B_{\max} in both cases (figure 22, a) (A_H , left: 41.6 %, $t = 2.62$, $p = 0.02$; A_H , right: 56.5 %, $t = 3.07$, $p = 0.008$; A_L , left: 20.8 %, $t = 2.22$, $p = 0.042$; A_L , right: 24 %, $t = 2.3$, $p = 0.036$, $df = 15$ for all). K_D values were not significantly altered in the hippocampus. ANOVA also revealed significant hemispheric interactions for A_H B_{\max} as well as both A_L B_{\max} and K_D in the AIv (A_H , B_{\max} : $F_{1,1} = 466.8$, $p = 0.029$; A_L , B_{\max} : $F_{1,1} = 1110$, $p = 0.019$; A_L , K_D : $F_{1,1} = 43000$, $p = 0.0001$). This resulted from coupling of significant increases in B_{\max} in the left AIv with

decreased B_{max} in the right (figure 22, a), or a decrease in A_L KD in the left AIv with an increase in the right. Alterations in the right AIv were non-significant (A_H , B_{max} : 45.8 % increase, $t = 2.71$, $p = 0.016$; A_L , B_{max} : 22.1 % increase, $t = 2.9$, $p = 0.011$; A_L , KD: 31.8 % decrease, $t = 2.29$, $p = 0.037$, $df = 15$ for all).

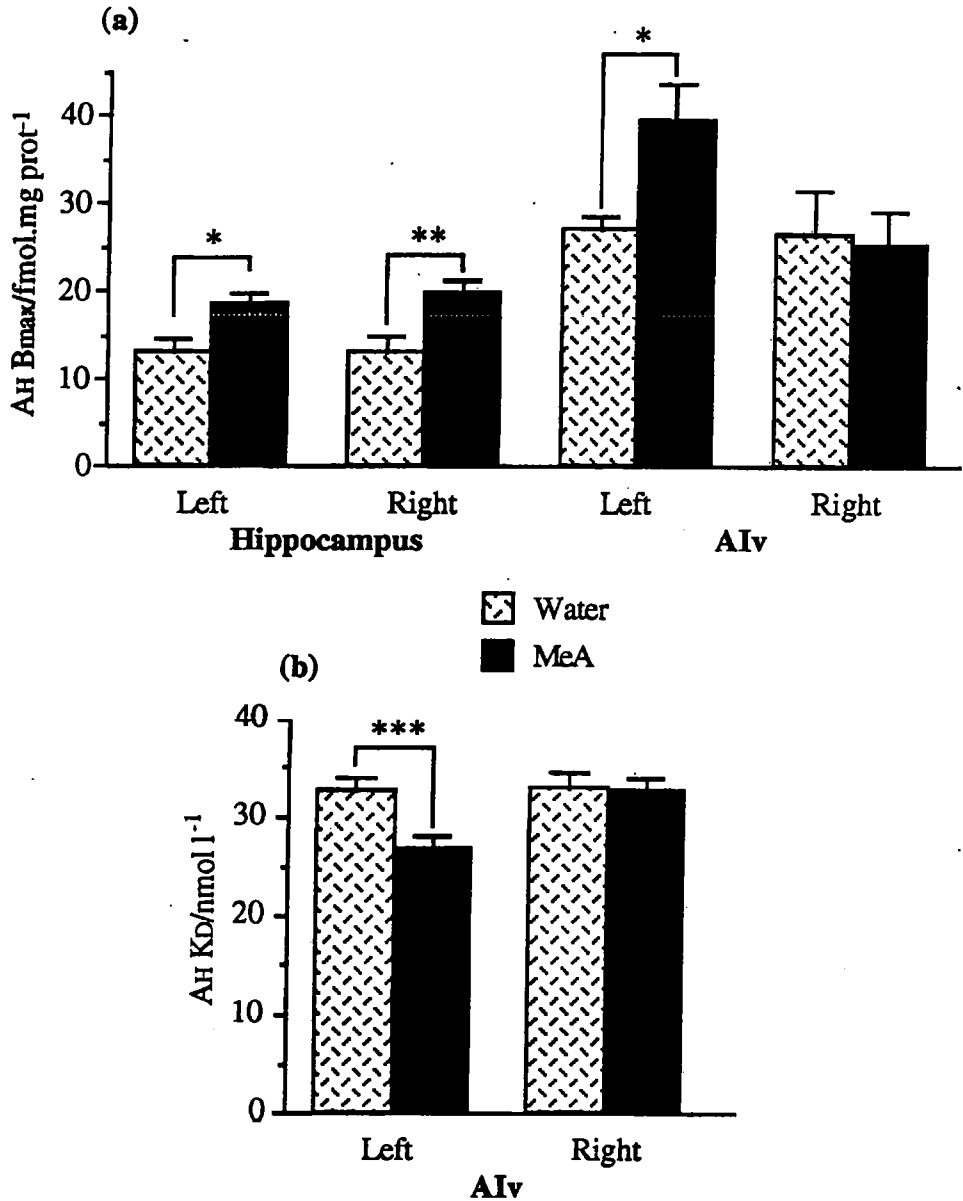


Figure 22: Alterations in (a) B_{max} for the high affinity component (A_H) of NMDA sensitive [3H]L-glutamate binding and (b) KD for A_H CPP sensitive [3H]L-glutamate binding, 3 h after passive avoidance training for MeA ($n = 8$) compared to water controls ($n = 9$). * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. Error bars represent standard error of the mean.

The only significant alteration in CPP sensitive binding revealed by ANOVA was in the A_H K_D , in the AIv ($F_{1,15} = 4.52$, $P = 0.049$). t-tests demonstrated that this was due to a significant decrease in A_H K_D in the left AIv (figure 22 (b), 17.8 %, $t = 4.23$, $p = 0.0007$, $df = 15$).

10.2.4: Discussion

In this study at 3 h post-training, Scatchard analysis revealed two components of both NMDA and CPP sensitive binding, and demonstrated that the A_H K_D for NMDA sensitive binding was significantly less than that for CPP sensitive binding in all regions, whereas the A_L K_D for NMDA sensitive binding was significantly higher than that for CPP sensitive binding. The presence of non-specific binding as an explanation for the two component binding curves can be ruled out as LIGAND corrects for this, and a two step/three component binding reaction seems highly unlikely given the well known structure and properties of the NMDA receptor. Two possible explanations for these findings, therefore, remain (section 6.1). One is that NMDA and CPP bind to a homogeneous population of receptors with different affinities, but with negative cooperativity. If this were true, however, it would be expected that the pattern of training related alterations in the binding parameters of NMDA and CPP sensitive binding would coincide. This was clearly not the case, and combined with the significant differences between regional NMDA and CPP sensitive binding (section 10.1), these data clearly indicate the presence of a heterogeneous population of NMDA receptors in the chick forebrain, comprising at least two subtypes.

It has been accepted for some time that multiple NMDA receptor subtypes are present in the mammalian brain^{34,136} (section 2.1.2), and indeed a number of isoforms have been identified by molecular cloning^{34,40,189}. How their various properties relate to their functions in different brain regions and how those functions differ, however, remains to be ascertained. The two major NMDA receptor subtypes present in the rat forebrain were referred to in chapter 1 (section 2.1.2) as nNMDA, which predominate in the striatum and

dorso-lateral septum and have a higher affinity for agonists such as NMDA and L-glutamate than for antagonists, and cNMDA which predominate in the thalamus and have a higher affinity for CPP and related antagonists than for agonists^{34,136}. nNMDA receptors also have a higher affinity for L-glutamate ($EC_{50} = 0.34 \pm 0.03 \mu\text{mol l}^{-1}$ in the striatum) and a lower threshold of activation than cNMDA receptors ($EC_{50} = 0.84 \pm 0.15 \mu\text{mol l}^{-1}$ in the thalamus)¹³⁵.

Chick NMDA receptors would appear not to be identical to those in the rat. Their affinities are much higher (this is true for [³H]L-glutamate binding in general, rat $K_D = 285 \text{ nmol l}^{-1}$ ²²⁹, chick $K_D = 100 \text{ nmol l}^{-1}$ ¹²⁵), and although similar in transmembrane topology, a large portion of the avian NR1 subunit (66 amino acids at the C-terminus) has no homology with that of the rat¹⁰⁸. Nevertheless, it seems that the two major NMDA receptor subtypes in the chick forebrain differ in a similar fashion to those of the rat. The receptor heterogeneity detected in this study, therefore, probably indicates that NMDA sensitive [³H]L-glutamate binds to putative nNMDA receptors with high affinity and to putative cNMDA receptors with much lower affinity, whereas the opposite is true of CPP sensitive [³H]L-glutamate.

The findings reported here confirm that bilateral increases in binding to NMDA receptors in the hippocampus, 3 h after passive avoidance training, result from an increase in the number of receptors present in the region (ie increased B_{max}), rather than increased affinity. Previously observed binding alterations in the TPO and neostriatum (section 8.2), however, were not reflected in the binding parameters found here, although B_{max} in the left AIv, a region not measured in the previous study, did increase. The high and low affinity B_{max} for NMDA sensitive binding increased in both the hippocampus and in the left AIv. The increases in $A_L B_{\text{max}}$, however, can be attributed to increased high affinity binding, as $A_L B_{\text{max}}$ is a measure of the number of receptors which contribute to both components and the % increases in $A_H B_{\text{max}}$ were all higher than those of $A_L B_{\text{max}}$. A training related decrease in K_D of the low affinity NMDA sensitive binding component in the left AIv was

also observed, and can be attributed to increased affinity of the cNMDA receptor subtype (see above).

The increased number of NMDA receptors observed in the hippocampus, in the present study, reinforces the existing evidence which suggests that the hippocampus has an important role in memory formation at a stage later than the IMHV or LPO. No such increases have been previously observed in the AIv, although a decrease in [³H]glycine binding in the AA (another archistriatal area) 3 h post-training was reported in section 8.2. It is not surprising, however, that an archistriatal area should have a role in memory formation. Archistriatal lesions have been shown to result in amnesia for passive avoidance training¹¹⁷ and the archistriatum has direct neuronal connections to the IMHV, LPO and hippocampus⁹⁴.

Alterations detected in CPP sensitive binding at 30 min post-training, were not observed at 3 h post-training. Assuming that the observed increase in CPP sensitive binding in the hippocampus at 30 min post-training was genuinely related to memory, this indicates that it was transient and that a temporal shift in the regional involvement of CPP sensitive receptors in memory formation, occurs between 30 min and 3 h post-training. In this study, the only alteration in CPP sensitive binding observed, was a training related decrease in K_D for the high affinity component, indicating increased affinity. This correlates with the decreased K_D of the low affinity NMDA sensitive binding component in the same region, and indicates that the high affinity component of CPP binding and the low affinity component of NMDA sensitive binding most probably represent the same receptor subtype (cNMDA), lending further support to the explanation of heterogeneous binding given above.

In summary, the data presented in this section strongly support the presence of at least two distinct NMDA receptor subtypes (equivalent possibly to nNMDA and cNMDA) in the chick forebrain and differential regional binding alterations between the two, after passive avoidance training, strongly suggest the possibility that they have distinct roles in

memory formation. The number of nNMDA receptors increases bilaterally in the hippocampus and in the left AIv, whilst the affinity of cNMDA receptors also increases in the left AIv, 3 h after passive avoidance training.

10.3

Alterations in the equilibrium binding parameters of NMDA receptors, 6.5 h after passive avoidance training.

Introduction: The data presented in sections 10.1 and 2 and in sections 8.1 and 2 clearly indicate that increases in the number of NMDA receptors present in specific forebrain regions, are important correlates of the early stages of memory formation, but whether such NMDA receptor up-regulation continues to be a key process at a later stage in memory formation is unclear. 6.5 h post-training is a time point which lies within the period during which processes involved in the second wave of memory formation occur in the IMHV¹⁷⁷. The experiment reported here investigates whether alterations in NMDA sensitive [³H]L-glutamate binding occur at this time point

10.3.1: *Methods*

Day old chicks were reared, housed and trained under the conditions described in chapter 2 (section 5.1). 40 chicks were pre-trained, trained, tested and the brains of 18 (9 W and 9 MeA) were processed exactly as described in section 10.1.1 for W/MeA chicks, except that chicks were tested and killed 6.5 h post-training. 42 sections were cut from each of the anterior and intermediate forebrain locations (figure 3, a and d), in each brain.

23 anterior sections (one triplicate and 10 duplicates) and 25 intermediate sections (one triplicate and 11 duplicates), were selected from each of the 18 brains for NMDA sensitive [³H]L-glutamate binding. Fourteen sections from each location of each brain were set aside for the [³H]AMPA binding experiment reported in section 11.1. The remaining

sections were used for scintillation counting and test strips (see chapter 2 sections 5.3.4 and 5.3.6).

Pre-incubations and incubations were performed as described in sections 5.3.3 and 10.1.1. NMDA sensitive [^3H]L-glutamate binding assays to anterior and intermediate sections were performed in separate sessions. Prior to the incubation of anterior sections, 10 concentrations of NMDA were made up by 75 % serial dilutions, (top: $400\ \mu\text{mol l}^{-1}$, 1 ml volumes) to each of which, 1 ml of $200\ \text{nmol l}^{-1}$ [^3H]L-glutamate was added, giving final displacer concentrations of 200, 50, 12.5, 3.125, 0.78, 0.195, 0.049, 0.012, 0.003 and $0.0008\ \mu\text{mol l}^{-1}$, all in $99.4\ \text{nmol l}^{-1}$ [^3H]L-glutamate (accurate concentration determined by scintillation count, s.a = 111 dpm/fmol). For incubation of intermediate sections, 11 NMDA concentrations were made up by 66.67 % serial dilutions (top: $400\ \mu\text{mol l}^{-1}$, 1 ml volumes) to each of which, 1 ml of $200\ \text{nmol l}^{-1}$ [^3H]L-glutamate was added, giving final displacer concentrations of 200, 66.67, 22.22, 7.4, 2.47, 0.82, 0.27, 0.09, 0.03, 0.01 and $0.0033\ \mu\text{mol l}^{-1}$, all in $95\ \text{nmol l}^{-1}$ [^3H]L-glutamate (accurate concentration determined by scintillation count, s.a = 111 dpm/fmol). In both assays 2.8 ml of $200\ \text{nmol l}^{-1}$ [^3H]L-glutamate was added to 2.8 ml of TCB for total binding

In each assay, one triplicate from each of the eighteen brains (ie, 18 triplicates in all), were incubated with [^3H]L-glutamate. 10 further groups of anterior duplicates, or 11 groups of intermediate duplicates, were incubated with the above stated range of NMDA concentrations in [^3H]L-glutamate. Incubations were terminated as described in section 10.2.1.

After processing, sections were apposed to [^3H]Ultrafilm (a maximum of 54 sections per film) with pre-prepared brain paste standards (section 5.3.5) and developed as previously described (section 5.3.6). Approximate apposition times were estimated from scintillation counts as in section 10.2.1²¹⁹. The actual apposition time before development of autoradiograms was 66 days.

Densitometry of autoradiograms was performed as described in chapter 2 section 5.3.7. The binding density of NMDA sensitive [^3H]L-glutamate in 20 brain regions for total binding (see fig 3, b and d), or 14 regions for the equilibrium binding parameters (6 anterior, including: LPO, MN, LN, MHV, LHV and HA; and 8 intermediate: AIv, AId, PA, N, TPO, IMHV, Hp and APH) were measured separately in each hemisphere, in addition to whole section and hemisphere measurements. Net binding (ie, NSB subtracted from total binding) data were processed as described in section 10.1.1, and data for equilibrium binding analysis were processed as described in section 10.2.1. Scatchard analyses were performed on regional binding data from each individual brain, using EBDA/LIGAND. Net binding, Bmax and KD values were sorted by code into W and MeA groups for each region measured in each hemisphere. The means, standard deviations and standard errors of the mean were then calculated. The means of the decoded equilibrium binding data for whole section measurements (average of left and right hemisphere data) in W control chicks, were taken and analysed using EBDA/LIGAND to find the average KD and Bmax values for whole sections. Regional Hill coefficients were calculated in EBDA. Analyses of regional differences in net binding, Bmax and KD, between W and MeA chicks, were performed using ANOVA and t-tests, as previously.

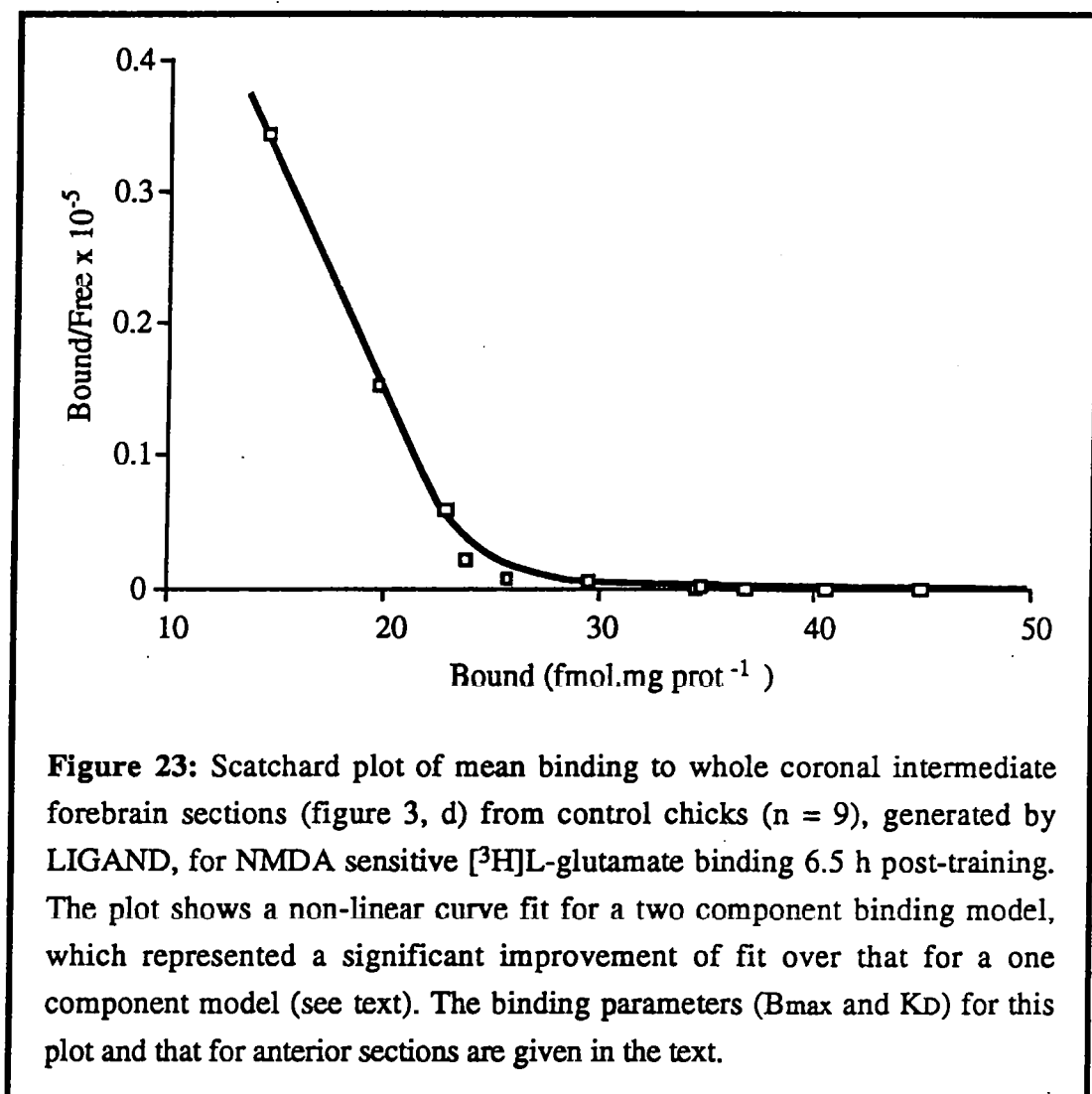
10.3.2: Results: Alterations in net NMDA sensitive [^3H]L-glutamate binding, 6.5 h after passive avoidance training

ANOVA revealed alterations in net NMDA sensitive [^3H]L-glutamate binding only in the IMHV ($F_{1,16} = 10.49$, $p = 0.005$). t-tests indicated that this was due to a significant bilateral decrease (left: 19.3 %, $t = 2.27$, $p = 0.038$; right: 22.9 %, $t = 2.94$, $p = 0.01$).

10.3.3: Results: Equilibrium binding parameters

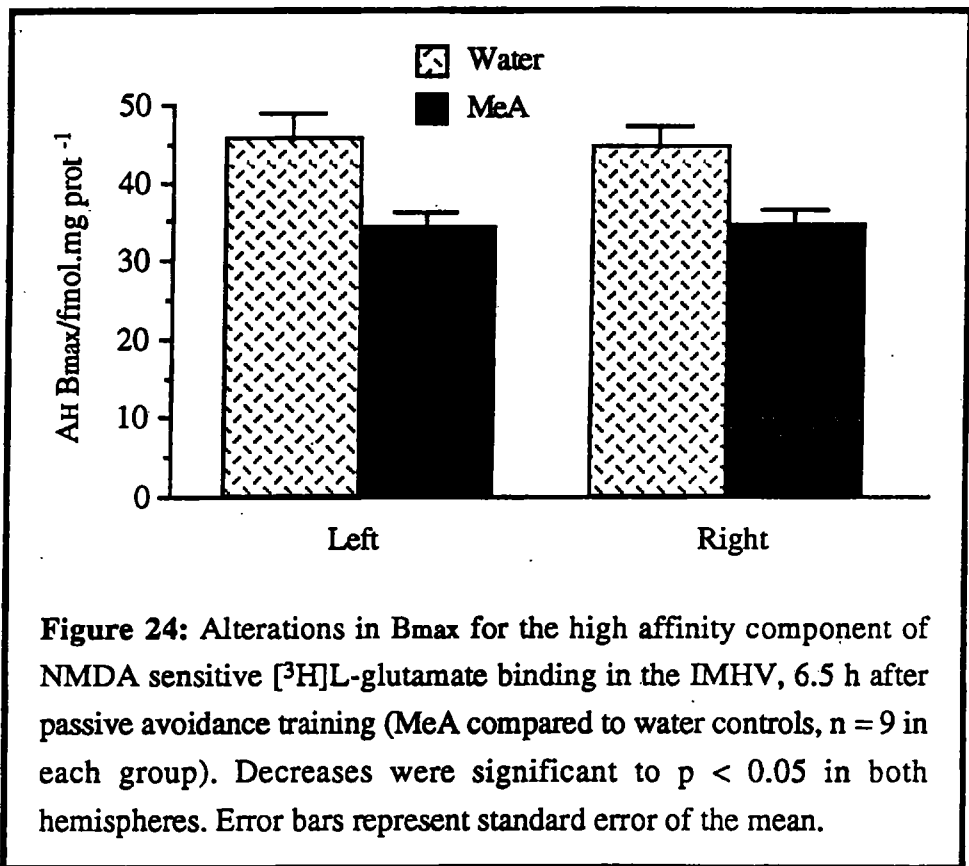
Analysis of average binding to whole sections from control chicks in EBDA/LIGAND, gave nH values of 0.72 ± 0.092 for binding to anterior sections and 0.425 ± 0.094 for binding to intermediate sections, both were significantly less than 1. ANOVA

performed by LIGAND during Scatchard analysis, revealed that a two site (curvilinear) model represented a significant improvement of fit compared to a one site model, for binding to both anterior and intermediate sections (anterior: $F_{7,5} = 23.4$, $p = 0.003$; intermediate: $F_{8,6} = 46.55$, $p = 0.00008$). The Scatchard plot generated by this analysis of binding to intermediate sections (with two component curve) is shown in figure 23. The equilibrium parameters of binding to whole sections from control chicks, as calculated by LIGAND, for NMDA sensitive binding to anterior sections were; A_H : $K_D = 20.4 \pm 3.2 \text{ nmol l}^{-1}$, $B_{\text{max}} = 22.1 \pm 3.38 \text{ fmol mg prot}^{-1}$; A_L : $K_D = 1.2 \pm 0.12 \text{ } \mu\text{mol l}^{-1}$, $B_{\text{max}} = 59.93 \pm 3.7 \text{ fmol mg prot}^{-1}$, and for binding to intermediate sections were; A_H : $K_D = 27.7 \pm 2.83 \text{ nmol l}^{-1}$, $B_{\text{max}} = 23.4 \pm 2.5 \text{ fmol mg prot}^{-1}$; A_L : $K_D = 2.3 \pm 0.21 \text{ } \mu\text{mol l}^{-1}$, $B_{\text{max}} = 53.5 \pm 4.6 \text{ fmol mg prot}^{-1}$.



10.3.4: Results: Alterations in the equilibrium parameters of NMDA sensitive [^3H]L-glutamate binding, 6.5 h after passive avoidance training

The only significant alteration observed in the equilibrium binding parameters was a bilateral decrease in $A_H B_{\text{max}}$ in the IMHV, reflecting a similar decrease in net binding (figure 24, ANOVA: $F_{1,16} = 15.86$, $p = 0.001$; (t-tests: left: 25.25 %, $t = 2.89$, $p = 0.011$; right: 22.8 %, $t = 2.73$, $p = 0.015$).



10.3.5: Discussion

The equilibrium binding parameters from the Scatchard analyses performed in this experiment, were broadly in line with those for NMDA sensitive [^3H]L-glutamate binding reported previously (section 10.2), although the $A_H K_D$ for binding to intermediate sections was rather high. The Scatchard analyses once again revealed two components of binding to NMDA receptors, further strengthening the evidence for at least two NMDA receptor subtypes in the chick forebrain.

These results indicate that at 6.5 h post-training a bilateral decrease in the number NMDA receptors present in the IMHV occurs. Given the body of evidence which implicates the IMHV in the second wave of memory associated processes which occur 6 - 9 h after passive avoidance training^{176,177}, it is not unexpected that such an alteration occurs at this time point. It may seem surprising that a decrease in the number of receptors should occur rather than an increase, but there is little evidence from species other than chicks to indicate that NMDA receptors are involved at such a late stage in memory associated processes. Evidence from studies of both mammalian and avian species implicates AMPA and metabotropic receptors in the maintenance of memory, at later time points after memory acquisition^{19,87,97,169,214}, so it is perhaps unexceptional that the number of NMDA receptors in the IMHV do not increase at at this time point. One possible explanation for the decrease in NMDA receptor number is that it is unconnected with regulation of the NMDA receptors, but is due to a decrease in the number of synapses. Such a decrease in synaptic number could result from processes such as selective synaptic stabilization, which lead to greater synaptic specificity.

10.4

NMDA receptor regulation and memory for passive avoidance training: general discussion.

The data presented in this chapter indicate the presence of at least two distinct NMDA receptor subtypes (presumed to be nNMDA and cNMDA) in the chick forebrain, which undergo memory specific, time dependant, alterations in their number or affinity (nNMDA: number; cNMDA: affinity and possibly also number) in different regions after passive avoidance training. Evidence for this includes two component Scatchard plots and Hill coefficients of approximately 0.5, for both NMDA and CPP sensitive binding (sections 10.2 and 10.3), indicating [³H]L-glutamate binding to a heterogeneous population of NMDA receptors. This is supported by significant regional differences in NMDA and CPP sensitive binding (section 10.1) and a differential pattern of memory related binding alterations (sections 10.1 and 10.2).

NMDA receptors are known to mediate a synaptic Ca^{2+} influx, which induces various processes related to memory formation^{21,27,165,177}. The observed increases in the number, or affinity, of receptors in specific forebrain regions must be a consequence of these processes. Although the actual mechanisms which lead to the increases remain enigmatic, there are a number of possibilities. Increases in the number of receptors at synapses could occur due to synthesis of new receptors (unlikely as early as 30 min post-training) or due to the freeing of previously occluded receptors¹¹⁴. Another possibility is, of course, that the observed increases are due to increases in the number of synapses. This latter idea is supported by the finding that the number of synapses in the right IMHV increases significantly 1 h post-training⁵⁹, which would suggest this to be a likely explanation for the increased number of cNMDA receptors 30 min post-training (as indicated by increased [³H]MK-801 binding - section 8.1, and by increased CPP sensitive [³H]L-glutamate binding detected in preliminary kinetic experiments). No such increases in synaptic number have been detected at such an early time point in either the left IMHV or either hemisphere of the LPO, suggesting that the more likely explanation for the observed alterations in the number of nNMDA receptors in these regions, is freeing of occluded receptors, as suggested by Lynch and Baudry¹¹⁴. The increases observed 3 h post training could be due to any, or a combination, of the three above possibilities. Increases in receptor affinity are easier to explain. It has been shown that the affinity of [³H]CPP binding to rat brain membranes is dependant on the lipid micro- environment of the receptor, and can be modified by PLC¹²². Also NMDA receptor affinity can be modified by PKC mediated phosphorylation⁵ and can be regulated in a complex manner by interaction of its multiple binding sites (section 2.1.2).

Increases in the number of NMDA receptors at existing synapses, such as those observed in the left LPO and IMHV 30 min post-training, must presumably enhance the influx of Ca^{2+} when the receptors are activated, and, therefore, the capacity of the synapse to undergo memory related plasticity. Increased NMDA receptor binding resulting from increases in synaptic number, although not affecting synaptic efficacy, would increase the

potential for such plastic change throughout the region involved, and also may indicate increases in the average number of excitatory synapses with afferent neurons, increasing the capacity of the afferent neuron to undergo plastic change. The detection of increased receptor numbers at a particular time point after passive avoidance training, however, does not necessarily mean that the receptors are active, or the processes which they mediate are taking place, at this time. Although the results of MK-801 binding experiments (section 8.1), in addition to some preliminary data, suggest that the number of NMDA (possibly the cNMDA subtype) receptors increases in the right IMHV 30 min post-training, increased L-glutamate release in this region was not detected until 3 h post-training (J.N.Daisley, unpublished data). This correlates well with the findings discussed in chapter 4 (section 9.1)³⁵ (Hölscher, unpublished) which show that pre-training MK-801 injections in the right IMHV do not result in amnesia up to 1 h post-training, but do at 3 h post-training. Increased L-glutamate release was detected 30 min post-training in the left IMHV, however, indicating that NMDA receptor activity is enhanced at this time point. Increased affinity of cNMDA receptors (eg, in the left AIv at 3 h post-training) also must result in enhancement of the Ca^{2+} influx through the receptor, but may act as a trigger which allows the receptor to initiate memory related processes in a region, rather than mediating some other function. Increased affinity also indicates a high likelihood of enhanced activity at the time of detection.

How the roles of these two NMDA receptor subtypes in memory formation differ, is not entirely clear, although it is likely that, as a result of their different properties, they trigger different memory related processes. The most obvious property which may be responsible for differences in the way that the two subtypes mediate such processes, is their affinity for L-glutamate. It is apparent from the results presented in section 10.3 that nNMDA receptors have a higher affinity for [^3H]L-glutamate than cNMDA receptors, as is the case in mammals¹³⁵. This is likely to mean that under normal circumstances, the Ca^{2+} influx through cNMDA receptors is smaller than that through nNMDA receptors. This may mean that different biochemical processes, the induction of which require different threshold Ca^{2+} concentrations, are initiated by the two receptor subtypes. Also, although mammalian

cNMDA receptors are just as sensitive to glycine as nNMDA receptors¹⁴³, this may not be the case for the analogous receptor subtype in the day old chick. The fact that injections of the glycine antagonist, 7-ClK, into the right IMHV had no effect on memory for the passive avoidance task in chicks tested 3 h post training, but that such injections of MK 801 did, suggests that this may be so. Glycine insensitivity may be another factor which results in differences in the role of cNMDA receptors in memory formation in the chick, in comparison to that of nNMDA receptors.

The importance of the temporal shifts in the regions where these alterations in binding occur will be discussed in chapter 7.

Chapter 6

The role of AMPA receptors in memory for passive avoidance training

11

AMPA receptors in memory for passive avoidance training: a quantitative autoradiographic study.

In chapters 4 and 5 it was shown that NMDA receptors play a crucial role in the early stages of memory formation after passive avoidance training in the chick, and that their up regulation is an important process which occurs during the first wave of synaptic plasticity in the IMHV and LPO¹⁷⁷, and a little later in the hippocampus and AIv. Longer term increases in synaptic efficacy, ie, increased ability of a synapse to elicit a response in an efferent neuron, however would be more likely to involve AMPA receptors. AMPA receptors are the most abundant excitatory receptor type in the vertebrate brain^{72,165} and their structure and properties are now well characterized¹⁸⁹ (chapter 1, section 2.1.1). Although the role of AMPA receptors in synaptic plasticity is less well established than that of NMDA receptors, their roles in LTP are known to be closely associated²¹ (chapter 1, section 3.1.1), and AMPA receptors have been implicated in longer term stages of memory and learning in mammals^{19,97,211,212} (chapter 1, section 3.2).

Until recently, no evidence for any involvement of AMPA receptors in memory for passive avoidance training in chicks had been found. Burchuladze and Rose (1991)³⁵ showed that injections pre-training, or five minute post-training, of the AMPA receptor antagonists CNQX, DNQX or NBQX, had no effect on memory for the passive avoidance task when chicks were tested 3 or 24 h later. Also, the results presented in chapter 3 (section 8) show that there are no changes in [³H]AMPA binding to chick brain sections 30 min or

3 h post-training. It must be noted, however, that after a classical conditioning paradigm in mammals, the K_D of binding to AMPA receptors was altered^{211,212}. Such alterations in AMPA receptor affinity could not be detected in the study reported in chapter 3. The results of a preliminary study of the equilibrium kinetics of [³H]AMPA binding, in MeA compared to water chicks (data are incomplete and are, therefore, not presented here), however, suggest that no changes in either the affinity or number of AMPA receptors occur 30 min after passive avoidance training. It has been reported recently that administration of DNQX 10 or 25 min after a similar passive avoidance task resulted in amnesia for the task 80 - 90 min post-training¹⁶⁹. It was, therefore, apparent that the role of AMPA receptors in memory for passive avoidance training required further investigation, and the experiments reported in this chapter were designed to do this.

11.1

Investigation of the equilibrium binding parameters of AMPA receptors, 6.5 h after passive avoidance training.

Introduction: In this experiment the effect of passive avoidance training on the equilibrium binding parameters of AMPA receptors was investigated 6.5 h post-training, in fourteen regions of the chick forebrain. The 6.5 h post-training time point is within the period during which the second wave of passive avoidance related biochemical processes in the IMHV occurs^{176,177}. The major aim of this experiment was to find whether any regional alterations in [³H]AMPA binding occur at this time point and if so, whether they can be attributed to changes in affinity or maximal binding. No changes in NMDA sensitive [³H]L-glutamate were found at 6.5 h except for a bilateral decrease in binding in the IMHV. Such a decrease could result from a reduction in the number of synapses and a finding of a similar decrease in the number of AMPA receptors in the IMHV, would strengthen this possibility.

11.1.1: Training and autoradiographic methods

The forebrain sections used in this experiment were cut from the same 18 chick brains as those used for the experiments described in section 10.3 (See section 10.2.1 for procedures up to pre-incubation). Fourteen sections were selected from each brain, and remaining sections were used for either the experiment reported in section 10.3, or scintillation counting and test strips (see chapter 1 sections 5.3.4 and 5.3.6).

Pre-incubations and incubations were performed as described in sections 5.3.3 and 10.1.1. [^3H]AMPA binding incubations for anterior and intermediate sections were performed in separate assays. Prior to the incubations, nine concentrations of [^3H]AMPA were made up by 50 % serial dilution, in addition to a solution of [^3H]AMPA in 1 mmol l^{-1} L-glutamate for NSB (accurate [^3H]AMPA concentrations were determined by scintillation counting, s.a = 121.4 dpm/fmol). All solutions were prepared in 5 mmol l^{-1} KSCN, in TCB. 5 mmol l^{-1} KSCN was used here in an attempt to improve the quality of [^3H]AMPA binding, in comparison to that in preliminary experiments (chapter 3, section 7.1.4).

Fifty-four anterior sections (1 triplicate from each brain of 9 W trained and 9 MeA trained chicks) were incubated with $255.86\text{ nmol l}^{-1}$ [^3H]AMPA. Eight further groups of sections (one section per brain in each group) were incubated with [^3H]AMPA concentrations: 104.54, 56.12, 25.1, 14.4, 7.82, 4.04, 2.03 and 1.13 nmol l^{-1} . Fifty-four further sections from the anterior brain location (again 1 triplicate from each brain) were incubated with $255.86\text{ nmol l}^{-1}$ [^3H]AMPA in 1 mmol l^{-1} L-glutamate solution for determination of non-specific binding. In the assay for binding to intermediate sections (using the same number of sections per brain for each concentration), the [^3H]AMPA concentrations used were: 323.01, 149, 75.4, 41.6, 21.44, 8.87, 5.43, 2.65 and 1.72 nmol l^{-1} . with $323.01\text{ nmol l}^{-1}$ [^3H]AMPA in the NSB solution. To terminate the incubations, all sections were washed three times in ice-cold buffer for 2 s, dipped in ice-cold distilled water to remove buffer salts and rapidly fan dried.

After processing, sections were apposed to [^3H]Ultrafilm (section 5.3.6) (a maximum of 54 sections per film), with pre-prepared brain paste standards (section 5.3.5). Apposition times were estimated as previously, from scintillation counts of spare sections incubated along with those for autoradiograms²¹⁹, as 20 days. The actual apposition time was 18 days. Autoradiograms were developed as described in section 5.3.6.

Densitometry of autoradiograms was performed as described in chapter 2 section 5.3.7. The binding density of [^3H]AMPA in fourteen chick forebrain regions (LPO, MN, LN, MHV, LHV, HA, AIv, AI_d, PA, N, TPO, IMHV, Hp and APH) were measured separately in each hemisphere, in addition to whole section and hemisphere measurements. Values were calculated in dpm for use in EBDA/LIGAND. Subtraction of NSB from [^3H]AMPA binding values was not necessary in this experiment, as no NSB to sections could be detected on autoradiograms.

Scatchard and Hill analyses were performed using EBDA/LIGAND. K_D and B_{max} values were calculated for [^3H]AMPA binding to each region of each individual brain. After decoding, the regional means, standard deviations and standard errors of the mean were calculated for each training group. Analyses of regional binding differences in B_{max} and K_D , between W and MeA chicks, were performed using ANOVA and t-tests. The mean of the decoded binding data for whole section measurements (average of left and right hemisphere data from intermediate sections) was then taken and this was analysed using EBDA/LIGAND to find the average K_D and B_{max} values for whole sections. Regional Hill coefficients were calculated in EBDA.

11.1.2: Results: Binding distribution

The binding distribution of [^3H]AMPA was similar to that reported in chapter 3 (section 8.1.2) (see table 8). Of the regions not previously measured, overall binding density in the AIv was second only to that in the hippocampus, but binding in the APH was more markedly lower than in the Hippocampus, and was also relatively low in the AI_d.

Table 8: Equilibrium parameters of [³H]AMPA binding to coronal chick forebrain sections, 6.5 h after passive avoidance training.

Parameter	High affinity Bmax				Low affinity Bmax			
Condition	Control (W)		Trained (MeA)		Control (ESA)		Trained (ESR)	
Hemisphere	Left	Right	Left	Right	Left	Right	Left	Right

(a) Anterior brain regions

LPO	63.5±28.8	74.1±21.1	36.3±3.8	70.2±15.3	757±407	468±157	1028±553	636±213
MN	28.5±1.9	41.4±5.5	28.7±0.8	35.8±2.2	306±161	544±110	416±219	642±87.0
LN	40.3±9.3	42.0±11.3	49.9±9.0	26.4±2.4	497±140	371±103	675±190	503±140
MHV	60.6±19.6	64.6±16.2	48.5±12.0	43.1±9.3	578±182	508±126	785±247	691±171
LHV	60.6±19.6	49.9±4.6	48.5±12	43.1±9.3	386±78.9	557±206	524±107	757±279
HA	28.5±1.9	41.4±5.5	28.7±0.8	35.8±2.2	537±211	351±80.7	730±287	477±128

(b) Intermediate brain regions

AIv	113±22.0	114±9.7	166±69.2	134±30.3	972±246	1003±315	1057±188	1123±403
AI _d	47.2±5.9	81.0±14.9	50.3±6.9	48.9±4.1	366±93.9	344±57.0	497±128	468±77.5
PA	61.4±10.1	<u>80.8±10.2</u>	68.7±8.5	<u>55.1±5.5</u>	390±52.2	460±93.3	530±80.0	626±127
N	47.5±6.6	73.7±12.8	52.5±4.8	52.2±7.2	315±84.6	404±69.3	428±115	548±94.2
TPO	65.4±9.4	145±83.3	66.1±11.8	144±58.4	379±77.2	523±130	515±105	711±177
IMHV	54.5±3	66.4±4.6	85.9±29	68.1±7.2	<u>777±89.0</u>	<u>746±64.2</u>	<u>543±49.2</u>	<u>550±47.8</u>
Hp	298±116	179±42.0	248±67.4	364±117	930±83	918±211	930±129	1176±286
APH	63.8±6.0	102±30.5	93.2±13.9	133±27.9	300±112	380±60.7	408±152	517±82.4

[³ H]AMPA	High affinity K _D				Low affinity K _D			
Condition	Control (W)		Trained (MeA)		Control (ESA)		Trained (ESR)	
Hemisphere	Left	Right	Left	Right	Left	Right	Left	Right

(a) Anterior brain regions

LPO	23.3±3.8	24.7±2.8	21.6±3.3	27.2±1.7	710±25.2	595±52.0	673±49.6	691±82.6
MN	16.2±2.3	24.8±2.5	15.6±0.9	26.0±2.4	620±49.5	673±71	694±65.7	714±92.3
LN	25.0±7.0	25.2±3.9	31.5±6.6	19.5±3.0	647±49.5	608±69.2	630±72.0	673±109
MHV	24.2±7.8	25.7±4.7	21.1±3.0	23.3±3.2	665±51.8	646±90.5	622±78.8	637±72.3
LHV	25.3±7.7	25.7±25.5	22.2±3.0	25.5±4.8	601±74.3	603±73.6	694±124	608±62.4
HA	16.2±2.3	24.8±2.5	15.6±0.9	26.0±2.7	611±73.2	648±67.1	629±117	609±62.4

(b) Intermediate brain regions

AIv	26.9±3.3	27.5±2.8	26.1±4.5	27.4±2.3	652±40.8	700±66.7	575±115	551±56.6
AI _d	17.0±2.9	27.0±3.0	19.3±3.9	22.1±2.5	614±54.0	598±83.0	714±142	567±78.6
PA	22.6±4.4	<u>24.4±1.4</u>	27.2±4.3	<u>19.6±1.5</u>	595±78.9	640±56.3	514±105	596±69.6
N	17.1±3.4	24.4±3.3	19.6±2.3	24.0±4.6	614±58.6	647±50.0	570±84.9	573±110
TPO	27.3±3.3	28.1±2.6	25.2±5.0	26.6±1.2	704±37.7	628±72.2	752±71.7	622±42.6
IMHV	20.0±2.5	26.0±2.0	19.6±1.3	23.0±1.3	<u>730±67.6</u>	<u>730±52.3</u>	<u>476±54.6</u>	<u>487±81.4</u>
Hp	26.5±1.8	24.1±2.3	26.5±1.8	23.9±1.3	581±94.6	549±83.2	507±70.7	560±67.8
APH	24.5±3.0	25.7±2.2	25.8±2.8	22.1±1.9	539±77.3	527±84.8	540±91.9	563±118

Bmax (fmol.mg prot⁻¹ ± standard error of the mean) and K_D (nmol l⁻¹ ± standard error of the mean) are means of calculated values for each individual brain.(from Scatchard analyses using EBDA/LIGAND). n = 9 for both water and MeA chicks. Significant differences between water and MeA trained chicks are underlined (see also figures 26 and 27)

On average binding levels in the Hp and AIv were very similar, but slightly higher in the Hp. In sections from some brains, however, binding was actually higher in the AIv than in the Hp. Binding was also relatively high in the hyperstriatum ventrale (including the IMHV) and the paleostriatal complex (including the LPO, but particularly in the PA). Apart from minor differences, the distribution observed here was similar to that observed in previous studies^{84,231} (section 8), except that binding levels in the HA were relatively low.

11.1.3: Equilibrium binding parameters

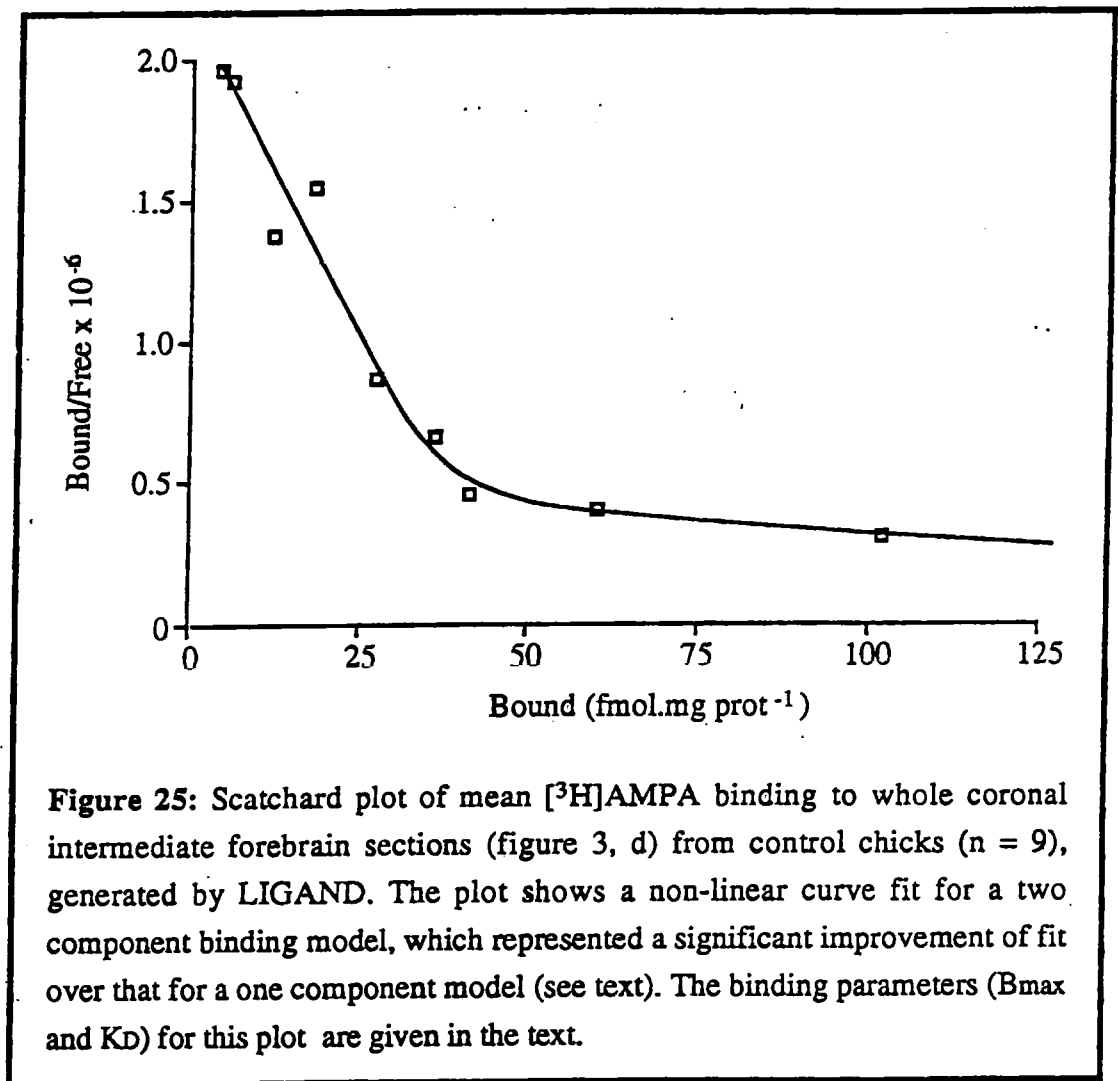
Scatchard and Hill analyses of data from whole intermediate sections revealed two components of binding with K_D values of 22.2 ± 1.1 nmol l⁻¹ (high affinity) and 685 ± 25 nmol l⁻¹ (low affinity), and associated B_{max} values of 46.9 ± 2 fmol.mg prot⁻¹ and 317.6 ± 32.4 fmol.mg prot⁻¹ respectively. Statistical analysis in LIGAND indicated that a two site model represented a significant improvement of fit compared with a one site model (ANOVA: $F_{6,5} = 6.73$, $p = 0.027$). The Hill coefficient, calculated in EBDA ($n_H = 0.77 \pm 0.023$) was significantly less than one, indicating more than one component of binding. The Scatchard plot generated by LIGAND is shown in figure 25.

Analysis of regional binding in the forebrain revealed similar two component binding parameters. There was some variation in these parameters, however, within the fourteen measured regions (control chicks only). Regional variation in B_{max} values reflected the [³H]AMPA binding distribution, the highest being in the hippocampus and the lowest in the AId. K_D values showed much less regional variation and were similar to those for binding to whole sections. Notable exceptions were the Hp and area perihippocampalis whose low affinity K_D values averaged 550 nmol l⁻¹.

11.1.4: Alterations in the equilibrium parameters of [³H]AMPA binding, 6.5 h after passive avoidance training

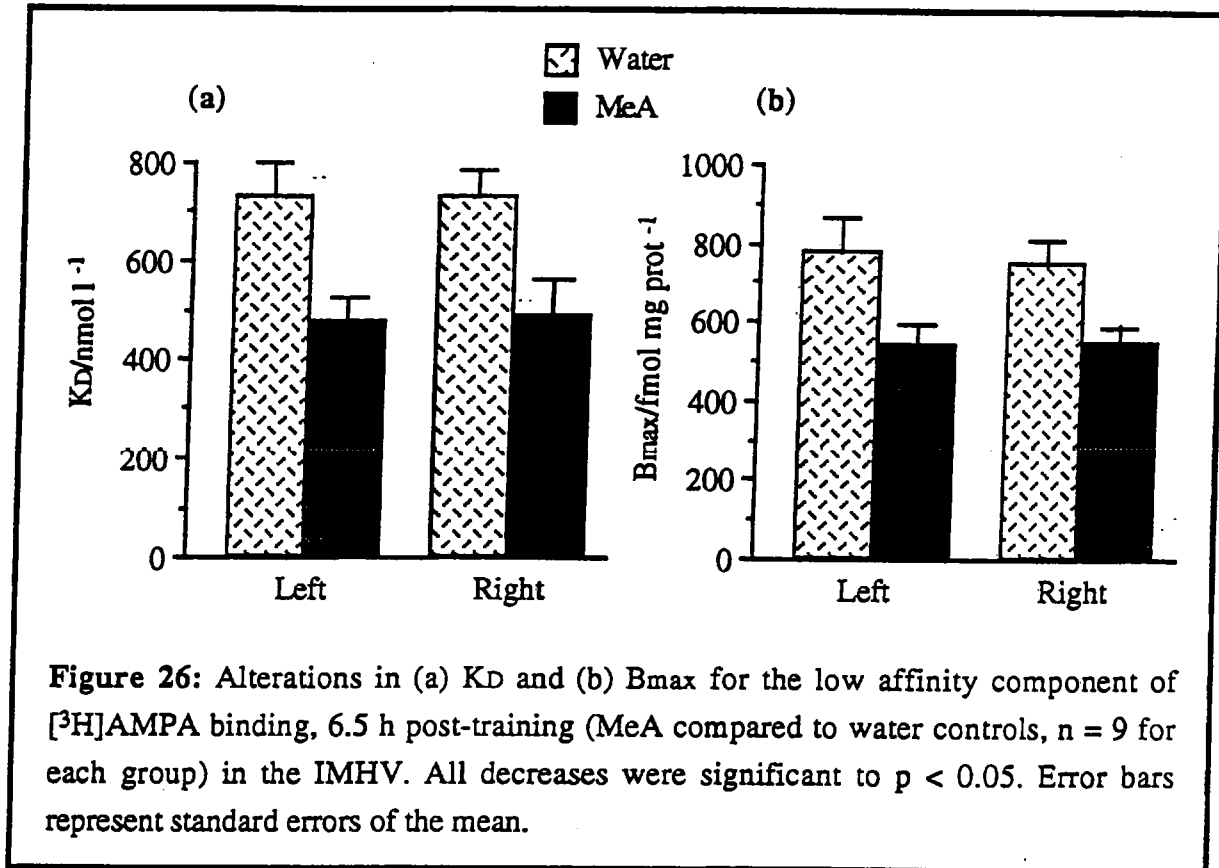
ANOVA revealed that the effect of training (MeA v W) on the low affinity binding parameters (K_D and B_{max}) was significant only in the IMHV (K_D : $F_{1,16} = 15.68$, $p = 0.001$,

B_{max} : $F_{1,16} = 10.65$, $p = 0.0046$). t-tests indicated that this was due to a significant bilateral decrease in K_D (figure 26 (a), left: 34.8% decrease, $t = 2.92$, $df = 16$, $p = 0.01$; right: 33.3% decrease, $t = 2.51$, $df = 16$, $p = 0.023$), accompanied by simultaneous decreases in B_{max} (figure 26 (b), left: 30.1% decrease, $t = 2.29$, $df = 16$, $p = 0.036$; right: 26.4% decrease, $t = 2.45$, $df = 16$, $p = 0.026$).



ANOVA revealed no significant training effects in any other region for either binding component, although there was a significant hemispheric interaction (K_D : $p = 0.045$, B_{max} : $p = 0.029$) in the high affinity binding parameters in the paleostriatum augmentatum (PA). This resulted from significant decreases in both K_D and B_{max} in the right PA accompanied by small increases in both parameters in the left (figure 27 (a), right K_D : 19.5% decrease,

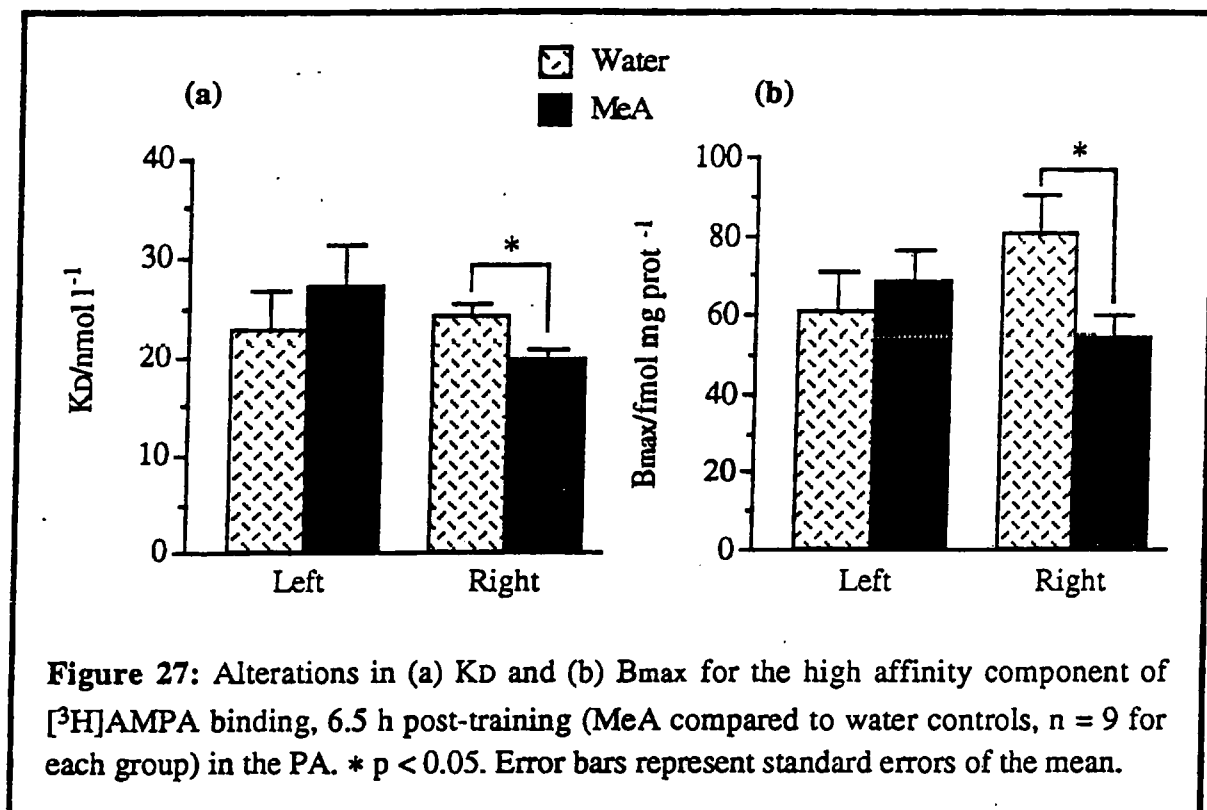
$t = 2.32$, $df = 16$ $p = 0.034$; figure 27(b), right Bmax: 31.8% decrease, $t = 2.21$, $df = 16$, $p = 0.042$.



11.1.5: Discussion

In this experiment, two components of [³H]AMPA binding were identified in all forebrain regions measured and these had KD values similar to those identified in previous studies using rat brain membranes⁸⁰. Here KD values for high affinity binding to whole chick forebrain sections were 22.2 ± 1.1 nmol l⁻¹, and 685 ± 25 nmol l⁻¹ for low affinity binding, whereas in rat Hall et al (1993)⁸⁰ found values of 23 ± 6 nmol l⁻¹ and 660 ± 90 nmol l⁻¹ respectively. A recent autoradiographic study in the chick also revealed two component AMPA displacement of [³H]CNQX binding to sections, with K_i values of 3 ± 0.5 nmol l⁻¹ and 5.4 ± 0.4 nmol l⁻¹ ²³¹. It is not necessarily the case, however, that the two components of [³H]AMPA binding found here represent only two distinct AMPA receptor subtypes. The multiplicity of AMPA receptor subunits that has been revealed in the

mammalian brain¹⁸⁹ suggests that if the situation is similar in the chick, each of the two binding components could actually represent a number of subtypes whose affinities are too similar to be distinguished. The similarity between the K_D values found here and those found by Hall et al would suggest that this may well be the case.



In chapter 5 it was shown that NMDA receptor up-regulation plays an important role in the early stages of memory formation for the passive avoidance task. The results of preliminary studies (chapter 3, section 8), however, show that AMPA receptors do not appear to be involved in the initial processes associated with memory formation. At 6.5 h post-training, however, the K_D of the low affinity component of $[^3\text{H}]\text{AMPA}$ binding decreases bilaterally in the IMHV (figure 26, a), indicating increased affinity of the receptors, which would enhance excitatory transmission and, therefore, synaptic efficacy. This is the first indication that a receptor type is involved during the second wave of memory associated processes in the IMHV, after passive avoidance training. It is not surprising, however, that increased AMPA receptor affinity should occur at a relatively late stage after passive avoidance training, considering that memory related alterations in the properties of

AMPA receptors have been shown to occur at a similarly late stage after mammalian learning (see Chapter 1, section 3.2). Memory associated decreases in the K_D of the low affinity [3H]AMPA binding component, have been demonstrated previously in the mammalian hippocampus, as little as 3 - 4 h²¹² and up to 48 h post training²¹¹ on the nictitating membrane classical conditioning paradigm in rabbits.

The finding of a decrease in the K_D of the high affinity binding component in the right PA (figure 27, a) is more unusual. The only passive avoidance training associated alterations previously shown in the PA have been of a morphological nature²⁰², and as far as is known this is the first time that memory associated alterations in the high affinity component of binding to AMPA receptors has been reported. The PA, however, is part of the avian paleostriatal complex (equivalent to the mammalian striatum which plays a key role in long term memory formation²⁰²) along with the LPO and the paleostriatum primitivum, so it is perhaps surprising that more memory associated changes have not been found in this region.

Although the post-synaptic mechanisms which result in increased AMPA receptor affinity have not yet been elucidated in the chick, it seems likely that protein kinases and/or phospholipases may be involved. In mammals, it has been shown that the affinity of [3H]AMPA binding to rat brain membranes can be modified by treatment with either PLC or PLA₂ which act on the lipid micro-environment of the receptor^{16,121,122}. In addition, it has been shown that AMPA receptor mediated responses are enhanced by cAMP dependant PKA, which can phosphorylate AMPA receptors, increasing their affinity¹⁰⁰ (section 3.1.1).

The fact that B_{max} for [3H]AMPA binding decreases bilaterally in the IMHV after training, indicates that although the receptor affinity increased, the number of receptors decreased. This means that the number of AMPA, as well as NMDA (section 10.4), receptors decreased in the IMHV 6.5 h after passive avoidance training, lending further support to the possibility that the observed decreases in binding are due to a training related

reduction in the number of synapses. An alternative explanation could be that increased L-glutamate release in the IMHV (such an increase has been demonstrated 6 h post training- J. N. Daisley, unpublished data) leads to a decrease in the number of glutamatergic receptors at individual synapses. A "dynamical cascade model of receptor regulation" based on evidence for such an effect in mammals was proposed in a recent review¹⁹². No such decreases in receptor numbers coupled to increased L-glutamate release, however, have been found previously in the chick brain (at 30 min post-training both L-glutamate release⁵³ and the number of NMDA receptors (chapter 5) were shown to increase in the left IMHV). Such an explanation, therefore, seems to be less likely than a decrease in the number of synapses.

In summary, the results presented here indicate that increased affinity of AMPA receptors, bilaterally in the IMHV and in the right PA, is associated with longer term memory (6.5 h post-training) for passive avoidance training in chicks. This is investigated further, from the point of view of behavioural pharmacology, in section 12.

12

AMPA receptors in memory for passive avoidance training: behavioural pharmacology.

Introduction: Results of autoradiographic experiments reported previously (section 8), in addition to the results of Burchuladze and Rose (1991)³⁵, who found that three different AMPA receptor antagonists have no effect on memory for the passive avoidance task when injected pre or 5 min post-training, indicate that AMPA receptors are not involved in the early stages of memory formation. In a recent study, however, when DNQX was administered 10 - 25 min after chicks were trained on a similar passive avoidance task, which tests discrimination memory, they were shown to be amnesic when tested 80 to 90 min post-training¹⁶⁹. This would suggest the possibility that AMPA receptors have a role in memory consolidation.

The series of experiments reported in this section were designed primarily to investigate whether blocking AMPA receptors in the IMHV during the second wave of memory associated processes, has any effect on memory for the passive avoidance task, considering that memory related increases in AMPA receptor affinity occur 6.5 h post-training (section 11.1). The antagonist chosen here was CNQX, which is more selective for AMPA receptors than DNQX²³. CNQX injections, similar to those reported by Burchuladze and Rose (1991)³⁵, were also performed pre- and 5 min post-training, but using a higher CNQX concentration (see below) and a shorter time between training and testing.

12.1

The effect of i.c injections of CNQX on memory for the passive avoidance task.

Chick rearing, housing and training conditions and procedures were as described in chapter 2 section 5.1. Injection procedures were as described in section 5.2.

12.1.1: Determination of dosage.

Pairs of chicks were placed in pens and after 1 h were pre-trained 3 times on a dry white bead, and then presented with a dry chrome bead to test pecking behaviour. Those chicks which did not show a natural tendency to peck were discarded. Chicks were then injected, bilaterally (5 μ l per hemisphere), with a range of CNQX concentrations (100 nmol l⁻¹ - 100 μ mol l⁻¹) in 0.9% sterile saline, and their behaviour 30 min later was compared to that of chicks similarly injected with 0.9 % sterile saline only. Chicks injected with CNQX concentrations between 100 nmol l⁻¹ and 1 μ mol l⁻¹, exhibited no behavioural deficits and their ability to peck at a dry chrome bead was not impaired compared to saline injected chicks. Chicks injected with 1 μ mol l⁻¹ CNQX, however, pecked noticeably less than saline injected chicks and appeared drowsy, whilst at CNQX concentrations higher than this, pecking was further decreased and behavioural abnormalities were apparent, including lack of coordination and drowsiness, head swaying and general aimless motion. Those injected

with 100 $\mu\text{mol l}^{-1}$ CNQX were very drowsy and had little motor control. 500 nmol l^{-1} CNQX, half the concentration at which debilitating effects first became noticeable, was considered to be an appropriate dose and was used in subsequent experiments.

12.1.2: Training and injection schedule

A series of seven experiments were performed, each consisting of two trials (20-30 chicks in each) from which results were pooled. In each trial chicks were pre-trained, as described in section 12.1.1, and injected (i.c) with 5 μl per hemisphere of either 500 nmol l^{-1} CNQX (in 0.9% sterile saline) or 0.9% sterile saline. In experiments where post-training injections were administered, chicks were trained 10 min after the third pre-training trial. In experiments where pre-training injections were administered, chicks were injected 10 min after pre-training was completed. Chicks were tested by a researcher who was unaware of the experimental history of the chicks, using a dry chrome bead. Those chicks which did not show a natural tendency to peck on pre-training, or failed to peck the training bead were discarded. The training and injection protocols for the seven experiments are shown in table 9.

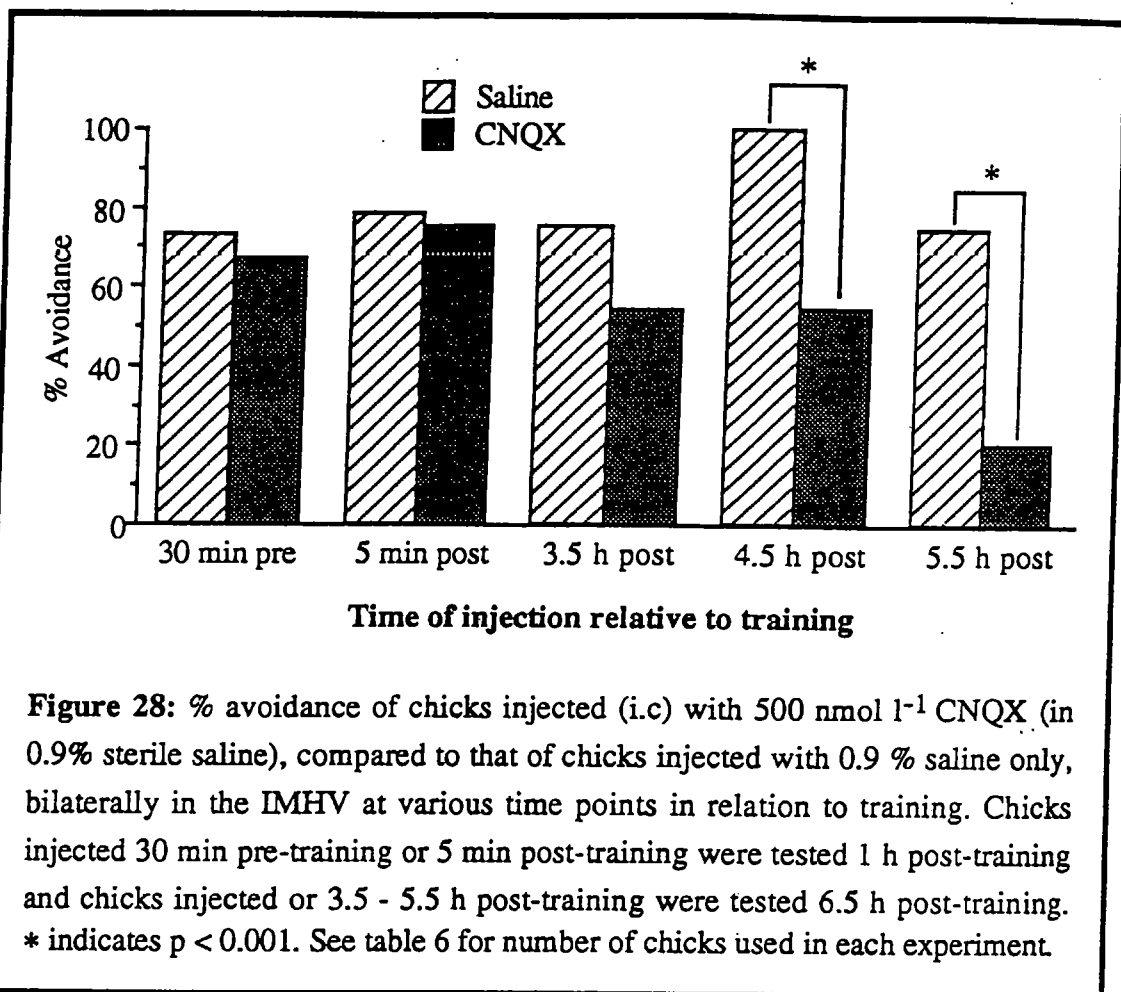
In addition a “visual discrimination” trial, where chicks were injected 5 min post-training and tested 1 h post-training, was performed in order to check for any non-specific effects which CNQX may have on pecking. Here, chicks were tested by presenting a yellow bead (Y1), then the usual chrome test bead 30 s later, then by re-presenting the yellow bead after a further 30 s (Y2). $2 \times 2 \chi^2$ tests, identical to those described in chapters 2 (section 6) and 4, were used to compare the number of chicks which avoided and pecked between Saline and CNQX treatments, but here they were also used to compare differences in avoidance or pecking between presentation of the chrome and yellow beads (for each presentation of yellow) for both CNQX and saline injected chicks.

Table 9: Experimental design. In all experiments half of the chicks were injected with 5 μ l of 500 nmol l⁻¹ CNQX (in 0.9% sterile saline) per hemisphere and half were injected with 5 μ l of 0.9% sterile saline. Where an odd number of chicks were used, the extra chick was injected with CNQX.

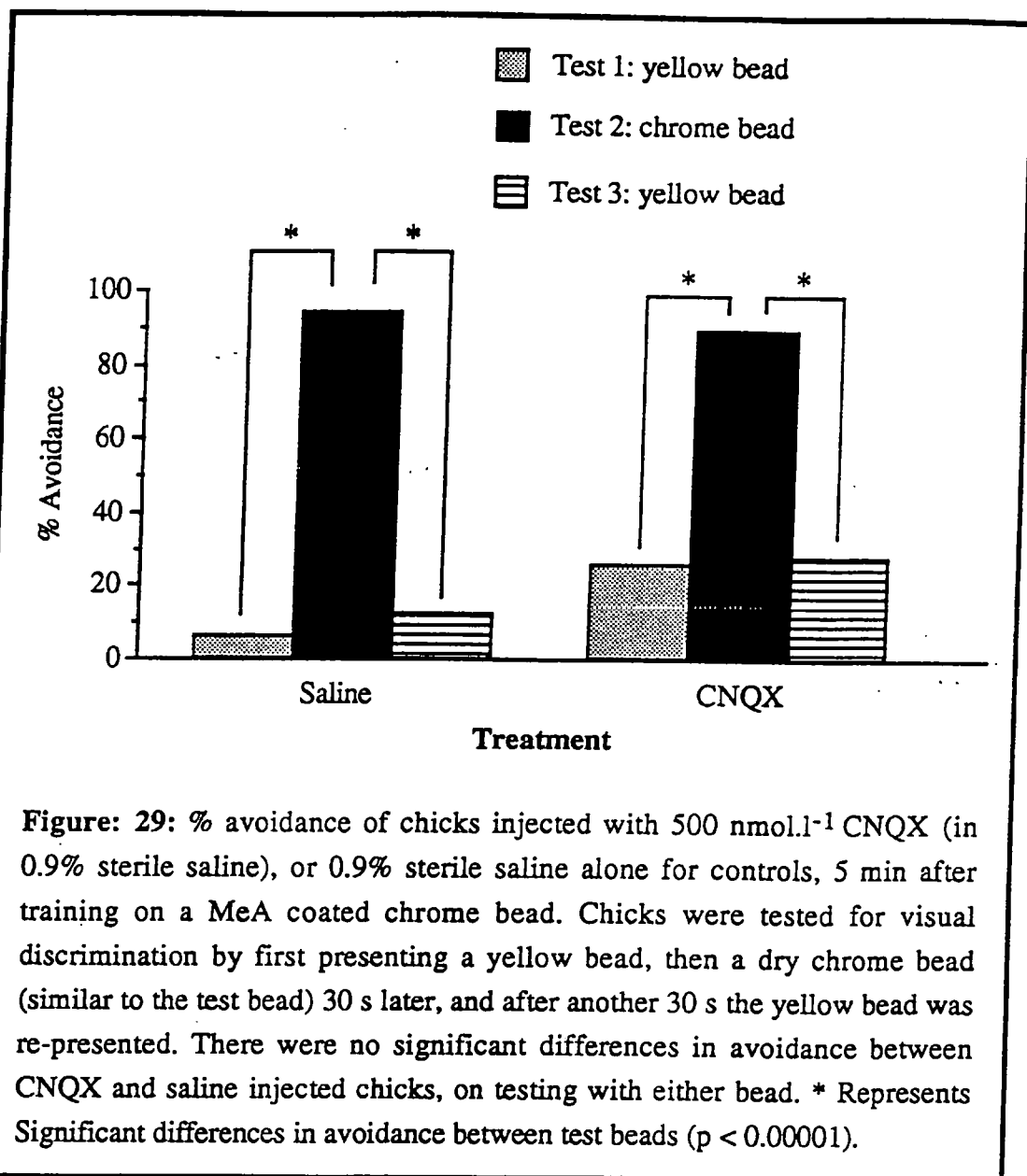
<u>Protocol</u> (No of chicks used in brackets)	<u>Hemisphere injected</u>	<u>Injection time</u> (Relative to training)	<u>Test time</u> (post training)
1 (a). (47)	Both	5.5 h post	6.5 h
1 (b). (48)	Left	5.5 h post	6.5 h
1 (c). (48)	Right	5.5 h post	6.5 h
2. (48)	Both	4.5 h post	6.5 h
3. (48)	Both	3.5 h post	6.5 h
4. (30)	Both	30 min pre	1 h
5. (47)	Both	5 min post	1 h

12.1.3: Results

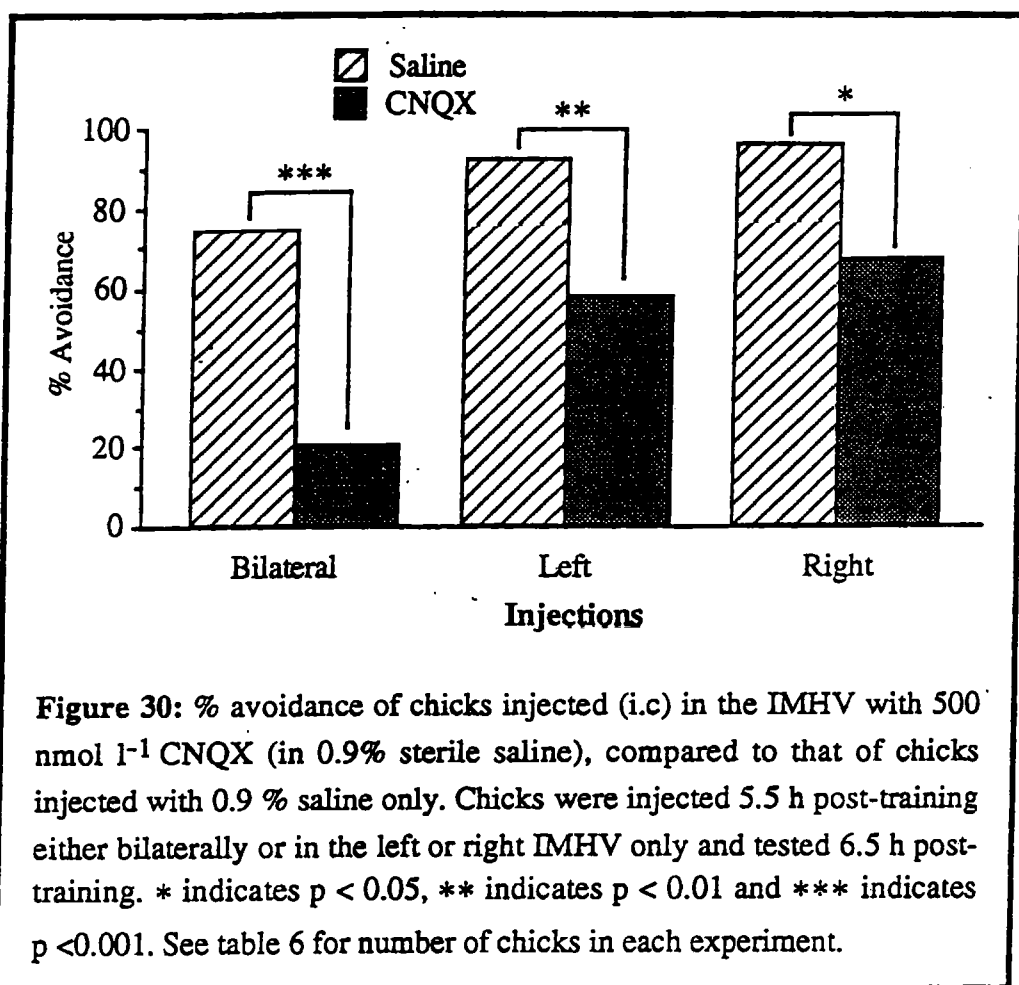
These results are presented in figure 28. When chicks were tested 6.5 h post training following bilateral i.c injections in the IMHV with CNQX, 4.5 or 5.5 h after training, significantly more CNQX than saline injected chicks failed to avoid the test bead indicating that CNQX has an amnestic effect on memory for the aversive experience. When injections were administered 4.5 h post-training 100 % of saline injected chicks avoided, whereas 54.2% of CNQX injected chicks avoided ($\chi^2 = 14.27$, $P < 0.001$). Injections of CNQX, compared to saline, made 5.5 h post-training, had a more pronounced effect: 74 % of saline injected chicks avoided whereas only 20 % of CNQX injected chicks avoided ($\chi^2 = 15.6$, $P < 0.001$). When chicks were injected 3.5 h and tested 6.5 h post-training the effect observed was non-significant (Saline: 75 % avoidance, CNQX: 54.2 % avoidance, $\chi^2 = 2.28$). No amnestic effects were observed when chicks were injected either pre-training or 5 min post-training and tested 1 h post-training.



No significant amnesic effects of CNQX, compared to saline, were observed in the visual discrimination trial (figure 29), when either the chrome or yellow bead was presented (Saline 94 % avoidance, CNQX: 89 % avoidance, on chrome test bead). The differences in avoidance between either presentation of the yellow bead and that of the chrome bead, however, were highly significant (Saline- Y1: 6 % avoidance, chrome: 94 % avoidance, Y2: 8 % avoidance, χ^2 (Y1 v chrome) = 24.5, χ^2 (Y2 v chrome) = 21.9, $p < 0.00001$ for both; CNQX- Y1: 26 % avoidance, chrome: 89 % avoidance, Y2: 28 % avoidance, χ^2 (Y1 v chrome) = 21.2, χ^2 (Y2 v chrome) = 20, $p < 0.00001$ for both). These data strongly suggest that CNQX has no non-specific effects on visual discrimination or pecking behaviour, under the conditions used.



The following results are presented in figure 30. Injections of CNQX in either the left or right hemisphere only, 5.5 h post-training (in chicks tested at 6.5 h) produced a significant amnesic effect, though much smaller than that when bilateral injections were administered at the same time point. 58.3 % of chicks injected with CNQX in the left hemisphere avoided, compared to 91.67 % for saline ($\chi^2 = 7.1$, $P < 0.01$), and 66.67 % of those injected with CNQX in the right hemisphere avoided compared to 95.8 % for saline ($\chi^2 = 4.92$, $P < 0.05$).



12.1.4: Discussion.

The results presented in this section, showing that CNQX injections into the IMHV pre or 5 min post-training, support the previous findings of Burchuladze and Rose (1991)³⁵, which indicated that NMDA receptors play an important role in the early stages of memory formation for the passive avoidance task, but that AMPA receptors do not. They also suggest, however, that activation of AMPA receptors in the IMHV is necessary around 6.5 h post-training, supporting the autoradiographic data which indicates increased AMPA receptor affinity at the same time point (section 11.2). This strongly suggests that in contrast to NMDA receptors, AMPA receptors are involved in the retention of a longer term phase of memory. Also, amnesia at the 6.5 h post-training time point, resulting from CNQX injections into the IMHV one or two h earlier, also shows that AMPA receptors are involved in the second wave of memory associated activity in the IMHV¹⁷.

The data from this experiment also indicates that fewer chicks show amnesia as the length of time between injection and testing increases (figure 28). This means that the effectiveness of CNQX as an amnestic drug (at the dose used) begins to decrease about 2 h after injection. It is possible, therefore, that CNQX blocks recall rather than the maintenance of long term memory. Although more work needs to be done to distinguish between these two possibilities, considering the fact that CNQX causes amnesia during a crucial period of memory related activity, as well as evidence from both hippocampal LTP and mammalian learning paradigms^{19,97,212,213}, it is more likely that it blocked maintenance of memory rather than recall.

The finding of a bilaterality of the altered [³H]AMPA binding affinity is also supported by the data presented here. Injections of CNQX to both left and right IMHV, at 5.5 h post-training, have a more marked amnestic effect on chicks tested 6.5 h post-training, than do either left or right unilateral injections (figure 30). It appears, therefore, that both the left and right IMHV are required for maintenance of longer term memory phases, and that AMPA receptors play a crucial role in this.

13

The role of AMPA receptors in memory for passive avoidance training: general discussion.

The data presented in this chapter show clearly that AMPA receptors play an important role in the retention of memory for the one trial passive avoidance task, at a relatively late stage after training, and previous data (section 8) show that they do not appear to be involved in the early stages of memory formation. It is known, however, that AMPA receptors play a crucial role in the induction of hippocampal LTP in mammals, where they mediate the necessary post-synaptic depolarization for NMDA receptor activation^{21,121}. It might be expected, therefore, that AMPA receptors would also play a role in the early stages of memory formation in chicks. The findings of preliminary autoradiographic experiments reported in section 8, that no changes in the binding properties of AMPA receptors occur 30

min after passive avoidance training, however, would argue against such involvement, unless AMPA receptors could play a role in the induction of memory related processes without any alterations in their binding properties being necessary. This seems unlikely since the behavioural pharmacology experiments reported in section 12.1, and those of Burchuladze and Rose (1991)³⁵, show that pre- or 5 min post-training injections of AMPA receptor antagonists have no effect on memory for passive avoidance training, confirming that AMPA receptors are not involved in the earliest stages of memory formation for this task in the chick. It is possible, therefore, that some other receptor type (the nicotinic acetylcholine receptor is a likely candidate²), may assist in the initial activation of NMDA receptors that is associated with memory formation in the chick.

The findings of Rickard et al¹⁶⁹, which show that non-NMDA receptors do play a role in memory for a passive avoidance task 80 -90 min post-training, indicates that AMPA receptors may be involved in memory consolidation. The low selectivity of DNQX, however, may mean that it is KA and not AMPA receptors which are involved. Also the paradigm was not identical to that used in our laboratories, and involved a discrimination between two coloured beads. It is possible, therefore that AMPA receptors may be involved in memory consolidation for visual discrimination, but not for the simple association of an unpleasant taste with a single bead. The results of the visual discrimination test, presented in section 12.1 (figure 29), however, suggest that this is not the case.

The results presented in sections 11.2 and 12.1 clearly show that AMPA receptors play a crucial role in the retention of longer term memory for passive avoidance training, and that this role involves increased synaptic efficacy resulting, at least partially, from an increase in the affinity of AMPA receptors. The 6.5 h time point at which this increased affinity was detected, and at which CNQX blocks memory retention, is similar to that at which protein and glycoprotein synthesis have been shown to occur in the IMHV¹⁷⁷. Moreover, alterations in a number of other physiological and biochemical factors have been shown to occur during the 6 - 8 post-training time window^{175,176,177}. Amongst the most

important of these, are significant increases in high frequency bursting activity in the IMHV 3-7 h after training, which were particularly large 6 - 7 h post-training⁷³. This directly indicates a memory related increase in synaptic efficacy, to which increased AMPA receptor affinity, such as that found here, would make a significant contribution.

In addition to their involvement in increased synaptic efficacy and, therefore the maintenance of memory, it is also possible that AMPA receptor activation could be involved in the initiation of the second wave of memory formation in the IMHV. It is apparent from the results presented in section 10.3 that NMDA receptors are not involved at 6.5 h post-training and, in contrast to the situation in mammals, a high proportion of post-synaptic AMPA receptors in the chick forebrain are Ca^{2+} permeable^{27,189}. It is a possibility, therefore, that AMPA receptors mediate the post-synaptic Ca^{2+} influx, which may be responsible for the induction of the second wave of memory associated biochemical processes. In this case CNQX injections at 4.5 or 5.5 h post-training would be blocking the induction of these processes.

In summary, the results presented in this chapter confirm that AMPA receptors are not involved in the early stages of memory formation, but show that an increase in their affinity is associated with the retention of memory for the passive avoidance task, during the crucial 6 - 8 h post-training time period, when the second wave of memory formation occurs in the IMHV. This increased affinity not only occurs bilaterally in the IMHV, but also in the right PA, and its importance in relation to memory retention is confirmed by the finding that AMPA receptor blockade by CNQX causes amnesia for the passive avoidance task at the same time point.

Chapter 7

The role of ionotropic L-glutamate receptors in memory formation after passive avoidance training

14

L-glutamate receptors in memory for passive avoidance training

At the end of chapter 1 the four major aims of this thesis were set out. These were (1) to "further establish the role of L-glutamate receptors in the formation and/or retention of memory for passive avoidance training", (2) to "clarify their mode of regulation by examining whether changes in their affinity or total number are affected by passive avoidance training", (3) to "examine the distinction between the roles of L-glutamate, and particularly NMDA, receptor subtypes" and (4) to "clarify which brain regions are involved in different stages of learning and memory". All of these aims, apart from the last, have been discussed in detail in chapters 4, 5 and 6. At this stage, however, it is worth re-examining the extent to which these aims have been achieved. Clearly the first two of these aims have been largely accomplished. The results presented in chapters 3 to 6, along with previous data³⁵, leaves little room for doubt that L-glutamate receptors are crucially important in memory for passive avoidance training. Also in chapters 5 and 6 their mode of regulation during memory formation was clarified. Here, in section 14.1, the involvement of L-glutamate receptors in memory and learning related synaptic plasticity, after passive avoidance training is discussed, with reference to the multiple trace hypothesis of memory formation^{69,128}. The main results of relevance to the third aim were discussed at length in chapter 5. It is clear from these that at least two NMDA receptor subtypes are present in the chick brain, and these are differentially regulated. In addition there appear to be differences in their regional localization and involvement after passive avoidance training. Moreover, the results presented in chapter 6 suggest that low and high affinity AMPA receptor subtypes are

involved in memory formation, in the IMHV and PA respectively, after passive avoidance training, although it is impossible to identify these under the conditions used, except in terms of their different affinities for [³H]AMPA. The fourth aim is discussed in more detail in section 14.2, where alterations in L-glutamate receptor regulation are considered as an indicator of the involvement of specific forebrain regions in memory formation at 30 min, 3 h and 6.5 h post-training.

14.1

The role of L-glutamate receptors in memory formation at the synaptic level

McGaugh's multiple trace hypothesis of memory formation (1968)¹²⁸, later applied to memory for passive avoidance training in the chick by Gibbs and Ng (1977)⁶⁹ is illustrated schematically in figure 31 (a). This model serves as a reasonably good guide to the sequence of events that occur after passive avoidance training, at the synaptic level in the IMHV. As mentioned in chapter 1, lesion studies as well as a variety of behavioural pharmacological studies have shown that the IMHV is involved in the early stages of memory formation^{35,75,89,175,176,200}. Immediately (0 - 5 min) after training, memory seems likely to be stored in the IMHV in the form of electrochemical neuronal activity. This type of memory trace (referred to in previous chapters as transient short-term memory) is equivalent to the buffer¹²⁸ or very short term memory⁶⁹ referred to in the multiple trace hypothesis (see figure 31 (a) and (b) for comparison). It is disruptable by transcranial subconvulsive electroshock in the first few minutes post-training, but rapidly decays and is not disruptable in this manner around ten minutes after training¹⁸⁰. By five min post-training the trace has decayed by 50 %¹⁵⁷ (see chapter 5) and this seems to be the time point where short-term memory proper has developed enough to supersede it.

The evidence presented in this thesis strongly suggests that the initial induction of the synaptic processes associated with short term memory formation, is dependent on NMDA receptors (chapters 3, 4 and 5), as would be expected from the NMDA receptor mediated induction of other forms of synaptic plasticity, eg, LTP²¹. Pre-training injections of NMDA

receptor antagonists such as MK-801 and 7-ClK, as well as 5 min post-training injections of MK-801³⁵, disrupt memory formation. In addition, however, the autoradiographically detected increases in binding to NMDA receptors, 30 min post-training (chapter 5), in conjunction with increased L-glutamate release at this time point⁵³, would suggest that a high level of NMDA receptor activity, relative to control chicks, continues to be important throughout the short term memory phase.

According to the multiple trace hypothesis, both short term and long term memory formation are initiated immediately after learning, but short term memory peaks and decays over a much shorter time course, and the processes involved with long term memory should not become apparent until later. This corresponds well with the double wave of processes in the IMHV, including increases in neuronal bursting activity⁷³ and glycoprotein synthesis¹⁸⁷, shown to be associated with passive avoidance training in our laboratories^{176,177}. The first wave of processes is initiated immediately after training and is detectable until about 1 h later, whereas the second wave occurs during a time window between about 6 and 8 h post-training^{176,177}. The evidence presented in this thesis for the involvement of L-glutamate receptors in memory formation, also reflects this double wave. The initial wave of processes seems to correspond with the short term memory trace in the multiple trace hypothesis, and involves NMDA receptors as discussed above (figure 31). No evidence for the involvement of AMPA receptors in the initial wave has been found (the amnesia resulting from DNQX injections, found by Rickard et al (1994)¹⁶⁹, was detected after the initial wave of memory formation is likely to have decayed, and is most probably due to disruption of intermediate term memory). It seems highly likely that the second wave of passive avoidance training related processes (eg, glycoprotein synthesis^{176,177}) are involved in long term memory formation, in which AMPA receptors would appear to have a crucial role, whereas NMDA receptors do not seem to be involved. CNQX injections administered 4.5 or 5.5 h post-training result in amnesia for the passive avoidance task at 6.5 h, and increased AMPA receptor affinity occurs at the same time point. This seems to be analogous to the situation in LTP where increased AMPA receptor affinity (and, therefore, synaptic efficacy) is necessary

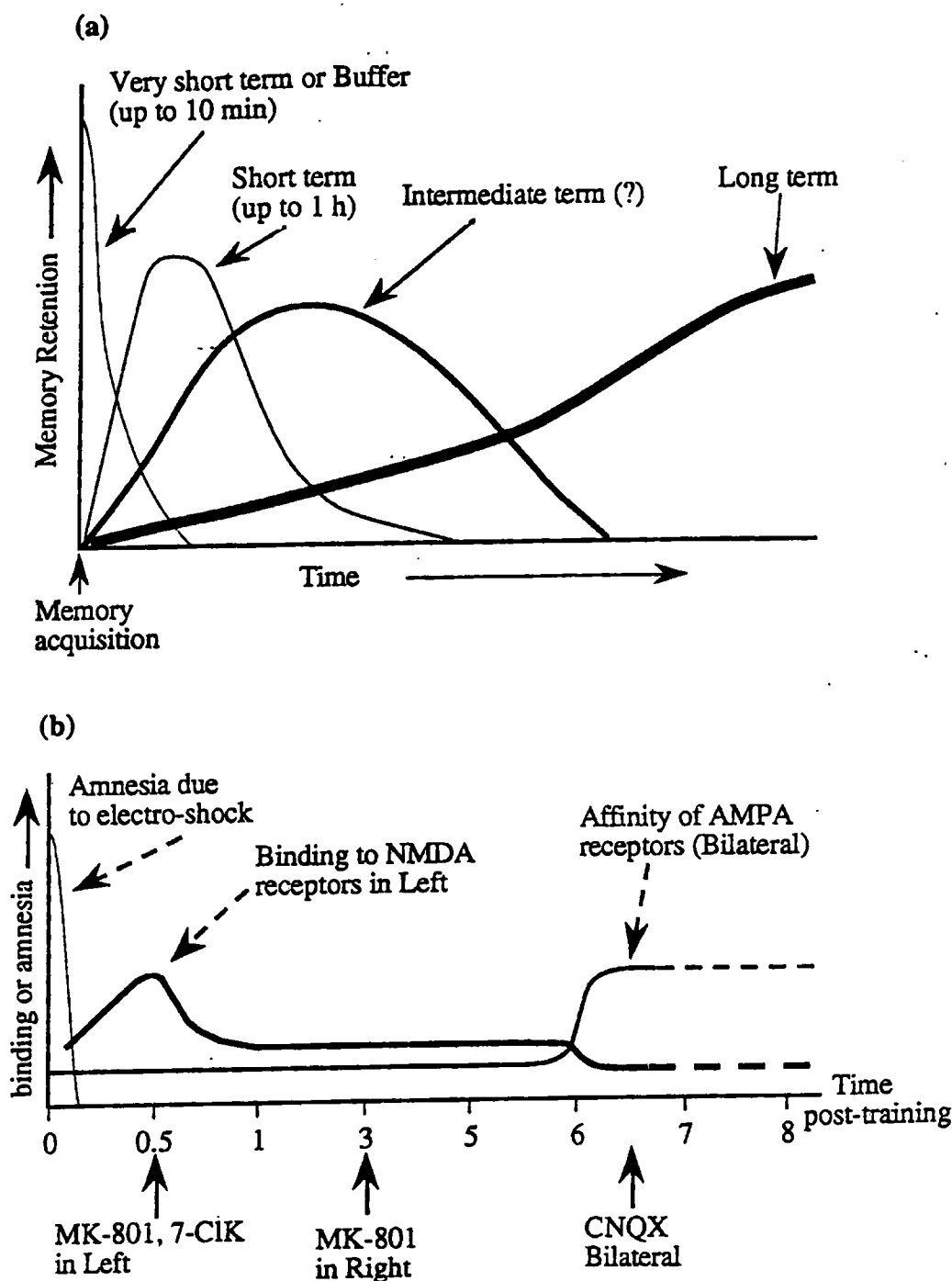


Figure 31: Multiple trace hypothesis. (a) a schematic representation of the multiple trace hypothesis of memory formation based on a diagram from McGaugh (1968). (b) Schematic representation of the time course of altered L-glutamate receptor properties in the IMHV after passive avoidance training (chapters 5 & 6), with the addition of the time course of amnesia after administration of transcranial electro-shock^{157,180}. In (b) solid arrows indicate post-training time points at which L-glutamate receptor antagonists are amnesic when injected into the IMHV.

for its long term maintenance^{21,121,213}. Increased L-glutamate release has also been detected around this time point in the right IMHV, but has not yet been investigated in the left hemisphere (J. N. Daisley, unpublished).

Although this discussion has concentrated on the role of ionotropic L-glutamate receptors in memory for passive avoidance training, it must be stressed that NMDA and AMPA receptors do not act in isolation. Augmentation and sensitization of receptors results from a complex cascade of processes which may well affect a number of different neurotransmitter-receptor systems. Other receptors whose involvement in memory for the passive avoidance task has been demonstrated, include: metabotropic L-glutamate receptors⁸⁷, muscarinic¹⁷⁸ and nicotinic² acetylcholine receptors, GABA receptors²⁹, δ -opioid receptors⁵⁰ and serotonin receptors². Also, altered levels of whole forebrain nor-adrenaline have been shown to occur after a passive avoidance task in the chick⁴⁹. In addition, glutamatergic transmission is known to interact indirectly with that mediated by a number of other transmitters including dopamine, serotonin¹¹¹ and GABA (the balance between synaptic excitation and inhibition)²¹. It is thus clear that altered synaptic efficacy due to memory formation must be under complex pharmacological control, mediated by interactions between a number of different receptor types and subtypes, whose involvement probably varies regionally, if not from synapse to synapse, and with time.

Although Gibbs, Ng and co-workers^{69,70}, as well as Rosenzweig and co-workers^{159,181} have provided clear evidence for the existence of intermediate term memory, as a phase distinct from short and long term memory, very little evidence for it has been found in our laboratory. Certainly no evidence for any intermediate term changes in the IMHV were found, during research for this thesis. It is possible, however, that processes involved in intermediate term memory occur in regions other than the IMHV. Temporal variations in the chick forebrain regions involved in memory after passive avoidance training, are discussed in section 14.2.

If one considers the results presented in this thesis, it is possible to speculate about the role of ionotropic L-glutamate receptors in the sequence of memory which leads to morphological modification in the IMHV (chapter 1, sections 3.3 and 4.3). The results of the behavioural pharmacology experiments presented in chapter 4, strongly suggest that NMDA receptors are involved in the induction of the cascade, probably via a post-synaptic influx of Ca^{2+} ¹¹. Increased post-synaptic Ca^{2+} concentration may act as a signal which activates various intra-cellular processes, including PKC translocation and activation, which results in the phosphorylation of a number of other proteins triggering further processes in the cascade¹⁷⁷, and synthesis of retrograde messengers such as NO and Aa which are likely to trigger processes involved in pre-synaptic plasticity^{88,89,90}. Memory related synthesis of proteins (eg, glycoproteins, receptor subunits, etc) via the immediate-early genes, c-fos and c-jun, may also be activated by PKC or by Ca^{2+} directly¹⁷⁷. Increases in the number of NMDA receptors (chapter 5) during the early stages of memory formation, may be a consequence of processes which occur during the cascade, and would further increase the post-synaptic Ca^{2+} concentration, activating additional processes which perhaps require higher levels of Ca^{2+} , and which may lead to modification of synaptic morphology²⁰². Increased affinity of AMPA receptors during longer term memory (chick AMPA receptors have relatively high Ca^{2+} permeability²⁷) may also be involved in the initiation of longer term processes which result in morphological changes, in addition to contributing to increased synaptic efficacy directly by enhancing excitatory transmission.

14.2

Temperal and regional aspects of memory for passive avoidance training

In addition to indicating the involvement and mode of regulation of L-glutamate receptors in memory for passive avoidance training, quantitative autoradiography also accurately indicates the regions where altered binding to receptors occurs. This serves as a good indicator of the forebrain regions that are involved in memory formation. Autoradiography was the technique used in pioneering experiments, which initially implicated the IMHV and LPO, as the major regions involved in chick learning and memory.

These involved 2-DG accumulation after passive avoidance training¹⁷⁸ and uracil incorporation after imprinting⁹⁴. Most work on the regional aspects of memory for passive avoidance training in our laboratories, however, has since involved lesioning techniques, where a brain region is physically damaged and the resulting behavior is observed.

Use of such lesioning techniques has shown that pre-training, but not post-training, lesions of the left IMHV result in amnesia, whereas post-training, but not pre-training, bilateral lesions of the LPO result in amnesia. In addition, post-training lesions of the right, but not left IMHV, after pre-training bilateral LPO lesions, resulted in amnesia^{75,175,176}. These results seemed to indicate that the left IMHV was involved in memory acquisition and the early stages of memory formation, and that the LPO was involved with memory retention. A hypothesis of memory formation, based on these results, involved migration of the memory trace from region to region^{175,176}. The memory would form in the left IMHV, and somehow migrate to the LPO via the right IMHV. This model did not, however, withstand further experimental scrutiny. Pre-training right IMHV lesions, which should block the "flow" of memory to the LPO, followed by a left IMHV lesion did not produce amnesia when the model predicted that it should¹⁷⁶. Also this hypothesis would predict that no memory related changes should be detected in the left IMHV at times later than that required for initial memory formation. Again, this is clearly not the case. The IMHV continues to be of importance in the second wave of memory formation 6-8 h post-training^{176,177} (chapter 6). Clearly, this model is rather simplistic and does not account for the complexity of inter-regional connections in the chick brain. Further lesion experiments, both in our lab and in others, which indicate the involvement of other regions (hippocampus¹⁸⁴ and archistriatum¹¹²), have further complicated the picture.

It seems likely that a more holistic, distributive model of memory formation may be closer to the true situation, and a good deal of data in support of such a model, including that reported in this thesis, has now been built up. In a distributive model, many, or even all, of the regions of the chick forebrain would be involved in memory formation and retention, but

the relative importance of individual regions would vary with time after training. Also individual regions could be responsible for different aspects of memory. The IMHV, for instance, could be involved in visual aspects of memory, eg, the colour, shape and orientation of the training bead. Colour discrimination experiments performed in our lab have shown that this is indeed the case¹⁷⁶ and also, alterations in the visual responsiveness of neurons in the IMHV have been shown to occur after imprinting³². The LPO, on the other hand, could be involved in memory for the unpleasant taste of methyl anthranilate, or in motivational aspects of learning. It forms part of the paleostriatal complex, which is the avian equivalent of the mammalian striatum²⁰², and has been shown to be involved in motivation¹⁴⁸.

One of the drawbacks with lesion studies is that they indicate only which regions are absolutely essential for a particular function, and do not necessarily indicate all of the regions which are involved at any particular time. A lesion which has no effect does not necessarily mean that the lesioned region is not involved in some aspect of the response. Altered binding to receptors, detected by quantitative autoradiography, however, is a relatively good indicator of a region's involvement at a given time point. A summary of post-training alterations in the binding properties of L-glutamate receptors in the chick forebrain, based on the results presented in chapters 5 and 6, is shown in figure 32.

At 30 min post-training, increased binding to nNMDA receptors was found in the left IMHV and left LPO (section 10.1), and alterations in the binding characteristics of cNMDA receptors were suggested by preliminary data in the right IMHV (section 8), and possibly the hippocampus (section 10.1). From this it is apparent that both the IMHV and LPO, and possibly the hippocampus are involved in short term memory. The continued involvement of the IMHV in the second wave of memory formation is apparent in increased AMPA receptor affinity at 6.5 h post-training, although no such alteration was detectable in the LPO. The LPO has, however, been implicated at this time point in other studies. Increased bursting activity⁷⁴ and increased L-glutamate release (J. N. Daisley, unpublished data) have both been

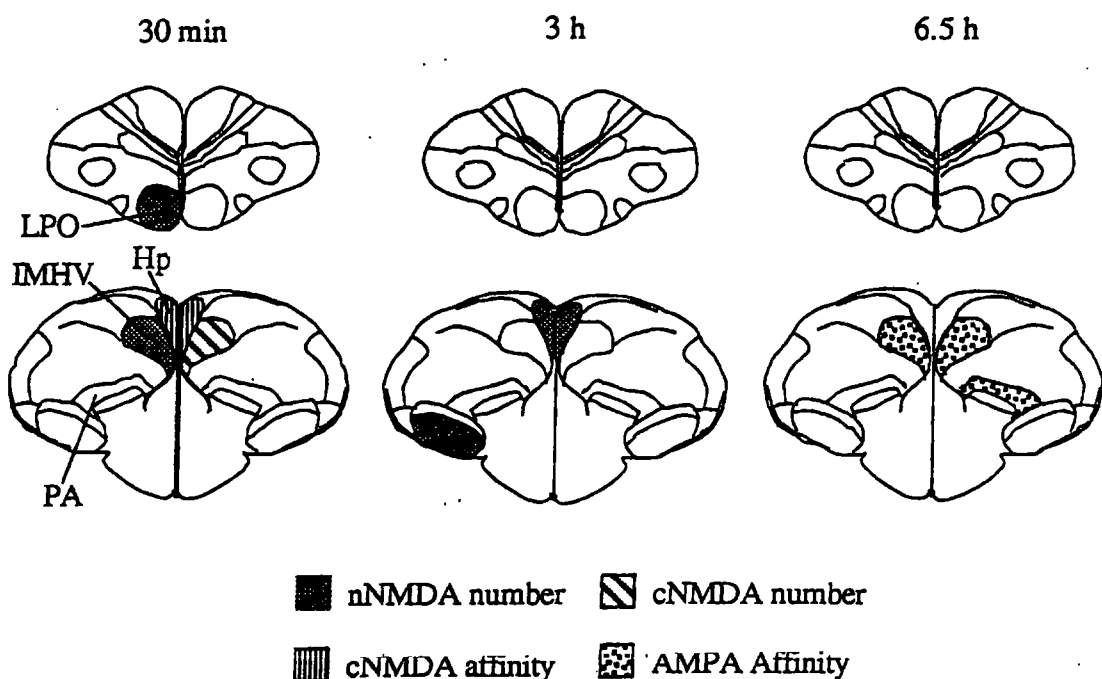


Figure 32: Overall regional increases in L-glutamate receptor properties. Schematic representation of anterior (top) and intermediate (bottom) level sections, showing regions where increases in the binding properties of L-glutamate receptors were found to occur at 30 min, 3 h and 6.5 h after passive avoidance training. The increases indicated at 30 min for cNMDA receptors are speculative, inferred from preliminary data only, and do not necessarily indicate hemispheric differences in the number of receptors present between the two subtypes.

detected in the LPO within the 6-8 h time window, and increased dendritic spine density¹¹³, in addition to other morphological alterations²⁰², has also been found in the LPO 24 h post-training. It is possible, therefore, that processes associated with the second wave of memory formation in the LPO involve a different type of receptor. MGluRs are likely candidates, although the LPO is rich in dopamine receptors¹⁴⁸ and these may also be involved. The affinity of AMPA receptors does, however increase in the right PA, a region which is closely associated with the LPO, and which may have a similar or complementary function. Increased synaptic bouton volume and altered synaptic vesicle numbers have also been found in this region 24 h after passive avoidance training^{202,203}.

At 3 h post-training, no alterations in the binding characteristics of L-glutamate receptors in either the IMHV or LPO were observed. Here the major alterations were in the

hippocampus and the left AIv. In section 14.1 it was mentioned that there is little or no evidence for any involvement of the IMHV in intermediate term memory, but that the involvement of other regions may be possible. These increases in binding to NMDA receptors in the hippocampus and left AIv may be an indication of this. If so this would also mean that increased NMDA receptor activity, in comparison to controls, is important in the formation of intermediate term memory in addition to long term memory. It has in fact been demonstrated that injections of the NMDA receptor antagonist D-AP5, into the centre of the forebrain at a depth of 3.5 mm (ie, in the region of the hippocampus) 10 or 30 min post-training, results in amnesia, the onset of which occurs around 1.5 h post-training¹⁶⁹. This suggests that D-AP5 blocks memory consolidation, perhaps by disrupting the formation of intermediate term memory in the hippocampus. In addition, pre-training lesions of the hippocampal/peri-hippocampal region have also been shown to result in amnesia at 3 h post-training¹⁸⁴ and bilateral archistriatal lesions have been shown to result in amnesia approximately 1 h post-training¹¹², on the passive avoidance task. The times at which amnesia were detected after these lesions are also consistent with the idea that the hippocampus and archistriatum are necessary for intermediate term memory.

It is also interesting to speculate about the particular aspects of memory for the passive avoidance task in which the archistriatum and hippocampus may be involved. The AIv has been considered as a homologue of the mammalian amygdala²³² and is thought to have "limbic" functions. It has been shown to be involved in fear and escape responses in a number of avian species^{77,115,163}. It is possible, therefore, that the AIv may be responsible for translating the association of the sight and unpleasant taste of the training bead with the disgust response, into a fear response when a chick encounters the test bead. The avian hippocampus has been most commonly associated with food storing behaviour¹⁰⁵, although its involvement in navigational learning in homing pigeons has also been demonstrated²⁰. MK-801 has been shown to impair the latter type of learning, again implicating NMDA receptors¹⁷⁰. Passive avoidance training is essentially a task which makes use of the ability of young chicks to discriminate between food and non-food items, and it is possible that the

hippocampus could be involved in this aspect of memory for the task. Another possibility is that it could be involved in memory for the spatial orientation of the bead.

No changes in the properties of L-glutamate receptors, however, were found in either the hippocampus or the AIv at 6.5 h post-training. This could indicate that NMDA receptor activation, during intermediate term memory formation in these regions, initiates plastic processes at synapses in which NMDA or AMPA receptors have no further involvement. The hippocampus and archistriatum are connected reciprocally to each other and both also have connections to the IMHV and LPO. It is possible, therefore, that even when the hippocampus and archistriatum are not apparently involved in memory, they could have an integrative role, facilitating communication between the IMHV and LPO, and perhaps other regions. Increased cNMDA receptor affinity in the hippocampus, 30 min post-training may be an indication of activity associated with this type of function.

In summary, alterations in the characteristics of binding to NMDA receptors in the left (nNMDA) and right (cNMDA) IMHV, left LPO(nNMDA) and hippocampus(cNMDA) 30 min after passive avoidance training, indicate that NMDA receptor mediated processes in these regions are involved in the early stages of memory formation. At 3 h post-training the involvement of the hippocampus and left AIv were indicated by increased binding to NMDA receptors, and at 6.5 h post-training increases in the affinity of AMPA receptors bilaterally in the IMHV and in the right PA indicated that enhanced glutamatergic transmission in these regions, is involved in the formation or retention of long-term memory. These results do not preclude the possibility that other regions may also be involved at the time points studied (particularly the LPO at 3 and 6.5 h, the Hp at 6.5 h, and the AIv at 30 min and 6.5 h), as their involvement may be dependent on processes in which ionotropic L-glutamate receptors do not take part.

14.3

Scope for further research

The bulk of work on the cellular correlates of memory formation after passive avoidance training, in our laboratories, has concentrated on the IMHV. It is apparent from the work presented in this thesis, and from other work inside and outside our laboratories, however, that more extensive investigation of other regions is merited, in order to obtain a complete picture of the spatio-temporal pattern of memory formation and storage. Although the involvement of the LPO in the consolidation and retention of memory is well established^{176,177}, the work in this thesis has clearly shown that, particularly in the right hemisphere, ionotropic L-glutamate receptors are not involved in this aspect of its function. Clearly further pharmacological work is required, perhaps concentrating on the role of dopamine receptors in motivational aspects of learning. Also the role of metabotropic L-glutamate receptors needs to be clarified, not only in the LPO, but also in other regions. It is also apparent from this and other studies that the hippocampus and archistriatum have crucial roles to play in memory for the passive avoidance task. It is clear that extensive studies of their roles need to be carried out in order to elucidate the aspects of memory in which these regions are concerned, their interactions with other regions, and their cellular correlates during the known phases of memory formation.

As far as ionotropic L-glutamate receptors are concerned, it still remains unclear how their binding characteristics are modified during memory formation. This is particularly true of NMDA receptor up-regulation where the number of receptors within a region increases. The major possibilities are increased synthesis of receptors at the genetic level or release of occluded receptors. This could be clarified using oligonucleotides which bind to mRNA coding for the recently cloned NMDA receptor subunits³⁴. These could be used in *in situ* hybridization experiments, to find whether any increases in NMDA receptor RNA synthesis is detectable, or could be injected to find whether blocking NMDA receptor expression results in amnesia.

Another very important question is: how important are the findings we make using passive avoidance training, in terms of memory and learning in general? In order to answer this question comparisons must be made between the data obtained using passive avoidance training and that obtained using other paradigms, both in chicks and in other species. To this end, we could develop and use other paradigms in our laboratory. This has already been done to a small extent with the appetitive pebble floor discrimination task for chicks (see chapter 1, section 4.1.3). In addition, however, closer cooperation with other laboratories where similar work is carried out using other paradigms, such as LTP and spatial learning in rats, is desirable.

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